UNIVERSIDADE DE LISBOA
Faculdade de Medicina Veterinária

INSIGHTS INTO THE DYNAMICS OF METHICILLIN-RESISTANT STAPHYLOCOCCI IN ANIMALS – A FOCUS ON *STAPHYLOCOCCUS PSEUDINTERMEDIUS* IN DOGS

NATACHA MONGE GOMES DO COUTO

ORIENTADOR: Doutora Maria Constança Matias Ferreira Pomba
CO-ORIENTADOR: Doutora Ana Maria de Jesus Bispo Varela Coelho

Tese especialmente elaborada para a obtenção do grau de Doutor em Ciências Veterinárias, especialidade de Clínica

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À minha família, aos meus amigos e ao Paulo

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Abstract – Insights into the dynamics of methicillin-resistant staphylococci in animals – a focus on *Staphylococcus pseudintermedius* in dogs

Staphylococci are a group of bacteria with clinical, agricultural, and economic importance because of their wide range of virulence factors and ability to become resistant to antimicrobials.

This thesis has pursued three main objectives:

I. Determine the frequency of methicillin-resistant *S. aureus* (MRSA) strains in several animal species, identify the characteristics of strains present in animals and comparison with human strains

MRSA nasal screening was performed in 71 horses and 307 calves, and the observed frequencies were 3% and 2%, respectively. Seventy-four MRSA isolated from 2001 to 2014 were characterized: fourteen *spa* types, three SCC*mec* types and three clonal complexes (CC) 5, CC22 and CC398, were found. Most isolates were multidrug-resistant. Fourteen MRSA CC398 strains had *qac* genes (13 *qacG* and 1 *qacJ*), while 4 isolates (three CC5 and one CC22) had insertions in the *norA* promoter gene. MRSA linages from pets (CC5 and CC22) harboured specific sets of virulence genes and a lower number of resistance genes than CC398 from livestock-animals.

II. Reveal antimicrobial/biocide susceptibility patterns/trends and resistance genes in methicillin-resistant staphylococci (MRS)

Several antimicrobial resistance patterns and genes were found in MRS from horses. Minimum bactericidal concentrations of biocides chlorhexidine acetate, benzalkonium chloride, triclosan and glutaraldehyde were lower than the recommended in-use concentrations for veterinary medicine, although two MRS carried plasmid-borne *qacA* and *sh-fabI* or *qacB* and *qacH*-like genes. An investigation on the evolution of resistance to 38 antimicrobials, corresponding mechanisms and molecular characteristics of 644 clinical *Staphylococcus* spp. isolates obtained from companion animals between 1999-2014 revealed resistance to the majority of antimicrobials and the number of *mecA*-positive strains increased significantly over time. Considering *S. pseudintermedius*, the methicillin-susceptible (MSSP) were genetically more diverse than methicillin-resistant (MRSP). All MRSP and two MSSP strains were multidrug-resistant, with several antimicrobial resistance genes identified. One MSSP isolate harbored a *qacA* and another a *qacB* gene. Three biocide products had high bactericidal activity (Otodine®, Clorexyderm Spot Gel®, Dermocanis Piocure-M®), while Skingel® failed to achieve a five log reduction in the bacterial counting.

III. Study of the pathogenesis of *S. pseudintermedius* in dogs

The *agr* type III predominated in MRSP. Five virulence genes were found in all strains and only *spsO* gene was significantly associated with MSSP. MSSP produced more biofilm on BHIB and BHIB+1% glucose than MRSP isolates. Several virulence genes encoding surface proteins and toxins were highly expressed in the MRSP strain (compared to MSSP). By whole proteome characterization of *S. pseudintermedius* through 2DE MALDI-TOF/TOF MS approach we were able to identify 367 unique proteins, of which 39 were surface proteins. By subsequent use of the serological proteome analysis (SERPA) approach we identified 4 antigenic proteins with promising features for vaccine development.

These results indicate that MRS were widely disseminated in the studied animal population, the environment and people in contact with these animals. The resistant trends and mechanisms detected in MRS strains are worrying and make animals a reservoir of important MRS clones and genes. Biocides are still a good therapeutic choice, even in the presence of efflux genes. Higher expression of virulence genes may play a role in the rapid and widespread of MRSP clones. Dogs are able to mount an IgG-response against *S. pseudintermedius* and the proteins identified by the immune system can in the future be used as vaccine candidates.

Keywords: *mecA*, staphylococci, methicillin-resistance, animals, vaccine

Resumo – Estudo da dinâmica de estafilococos meticilina-resistente em animais – um foco no *Staphylococcus pseudintermedius* em cães

Os estafilococos são um grupo de bactérias com importância clínica, agrícola e económica devido à ampla gama de fatores de virulência e pela sua capacidade de se tornarem resistentes aos antimicrobianos.

Esta tese debruçou-se sobre três objetivos principais:

I. Determinar a frequência de estirpes *S. aureus* meticilina-resistente (MRSA) em diversas espécies animais, identificar as características das estirpes presentes em animais e comparar com estirpes humanas

Colhemos zaragatoas de 71 cavalos e 307 vitelos para pesquisa de MRSA, e observaram-se frequências de 3% e 2%, respetivamente. Foram caracterizadas setenta e quatro estirpes MRSA isoladas entre 2001-2014: catorze tipos de *spa*, três tipos de SCC*mec* e três complexos clonais (CC) 5, CC22 e CC398, foram encontrados. A maioria das estirpes (74%) eram multirresistentes. Catorze estirpes de MRSA CC398 tinha genes *qac* (13 *qacG* e 1 *qacJ*), enquanto 4 (três CC5 e um CC22) tinham inserções no gene promotor *norA*. As linhagens de MRSA de animais de estimação (CC5 e CC22) tinham conjuntos específicos de genes de virulência e um menor número de genes de resistência do que as linhagens associadas aos animais de produção (CC398).

II. Revelar padrões/ tendências de suscetibilidade antimicrobiana/biocida e genes de resistência em estafilococos meticilina-resistente (MRS)

Foram encontrados vários padrões e genes de resistência antimicrobiana em MRS de cavalos. As concentrações bactericidas mínimas dos biocidas acetato de clorhexidina, cloreto de benzalcónio, triclosan e glutaraldeído foram menores do que as recomendadas em medicina veterinária, embora dois MRS tivessem os genes plasmídicos *qacA* e *sh-fabI* ou *qacB* e um *qacH*-semelhante. Uma investigação sobre a evolução da resistência a 38 antimicrobianos, mecanismos correspondentes e características moleculares de 644 *Staphylococcus* spp. clínicos obtidos de animais de companhia entre 1999-2014 revelou resistência à maioria dos antimicrobianos. O número de estirpes *mecA*-positivo aumentou significativamente ao longo do tempo. Quanto aos *S. pseudintermedius*, os meticilina-suscetível (MSSP) eram geneticamente mais diversos do que os meticilina-resistente (MRSP). Todos os MRSP e 2 MSSP eram multirresistentes, com vários genes de resistência identificados. Um MSSP tinha um gene *qacA* e outro um *qacB*. Três produtos biocidas tinham elevada atividade bactericida (Otodine®, Clorexyderm Spot Gel®, Dermocanis Piocure-M®), enquanto Skingel® não conseguiu atingir uma redução de 5 log na contagem bacteriana.

III. Estudo da patogenicidade de *S. pseudintermedius* em cães

O tipo III *agr* predominou nos MRSP. Cinco genes de virulência foram encontrados em todas as estirpes e só o gene *spsO* foi significativamente associado com MSSP. MSSP produziu mais biofilme em BHIB e BHIB + 1% glucose que as estirpes de MRSP. Vários genes de virulência que codificam proteínas e toxinas de superfície foram altamente expressos na estirpe MRSP (em comparação com MSSP).

Através da caracterização do proteoma total de *S. pseudintermedius* pela abordagem 2DE MALDI-TOF/TOF MS fomos capazes de identificar 367 proteínas únicas, das quais 39 eram proteínas de superfície. Posteriormente utilizámos a análise do proteoma serológico (SERPA) que identificou quatro proteínas antigénicas com características promissoras para o desenvolvimento de vacinas.

Estes resultados indicam que MRS estavam amplamente disseminados na população animal estudada, no ambiente e nas pessoas em contato com esses animais. As tendências de resistência e os mecanismos detetados em estirpes MRS são preocupantes tornando os animais um reservatório de clones MRS e genes. Os biocidas ainda são uma boa opção terapêutica, mesmo na presença de bombas de efluxo. Uma maior expressão de genes de virulência pode desempenhar um papel na rápida expansão de clones de MRSP. Os cães foram capazes de montar uma resposta IgG contra *S. pseudintermedius* e as proteínas identificadas pelo sistema imunológico podem, no futuro, ser utilizadas como candidatos vacinais.

Palavras-chave: *mecA*, estafilococos, resistência à meticilina, animais, vacina

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Abbreviations

1-DE – One-dimension electrophoresis
2-DE – Two-dimension electrophoresis
ABC – adenosine-triphosphate-binding cassette superfamily
ACME – Arginine catabolite mobile element
AIP – Autoinducing peptide
ATP – Adenosine triphosphate
BURST – Based upon related sequence type
CA-MRSA – Community acquired – methicillin-resistant *S. aureus*
CC – Clonal complex
cDNA – Complementary deoxyribonucleic acid
CLSI – Clinical and laboratory standards institute
CoPS – Coagulase-positive staphylococci
CoNS – Coagulase-negative staphylococci
CP5 – Capsular polysaccharide 5
CP8 – Capsular polysaccharide 8
DHPS – Dihydropteroate synthase
DNA – Deoxyribonucleic acid
dru – Direct repeat unit
HA-MRSA – Hospital-acquired/associated – methicillin-resistant *S. aureus*
HMM – Hidden Markov Models
IEC – Immune evasion cluster
IgG – Immunoglobulin G
IS – Insertion sequence
LA-MRSA – Livestock-associated – methicillin-resistant *S. aureus*
LC-MS/MS – Liquid chromatography coupled to Mass spectrometry/Mass spectrometry
MALDI-TOF/TOF MS – Matrix-assisted laser desorption/ionization-Time-of-flight/Time-of-flight Mass spectrometry
MATE – Multidrug and toxic compound extrusion family
MBC – Minimum bactericidal concentration
MFS – Major facilitator superfamily
MHC – Major histocompatibility complex
MIC – Minimum inhibitory concentration
MLS – Macrolides, lincosamides and streptogramins
MLS_B – Macrolides, lincosamides and streptogramins B
MLST – Multi-locus sequence typing
MRCoNS – Methicillin-resistant coagulase-negative staphylococci
mRNA – Messenger ribonucleic acid
MRS – Methicillin-resistant staphylococci
MRSA – Methicillin-resistant *Staphylococcus aureus*
MRSE – Methicillin-resistant *Staphylococcus epidermidis*
MRSP – Methicillin-resistant *Staphylococcus pseudintermedius*
MS – Mass spectrometry
MSCRAMM – Microbial surface components recognizing adhesive matrix molecules
MSSA – Methicillin-susceptible *Staphylococcus aureus*
MSSP – Methicillin-susceptible *Staphylococcus pseudintermedius*
nanoLC-ESI-MS/MS – Nano liquid chromatography - Electrospray ionization - Mass spectrometry/Mass spectrometry
min – minutes
NGS – Next-generation sequencing
PA-MRSA – Pets-associated – methicillin-resistant *Staphylococcus aureus*
PBP2a – Penicillin-binding protein 2a
PCR – Polymerase chain reaction
PIA – Polysaccharide intercellular adhesion
PFGE – Pulsed-field gel electrophoresis
PNAG – Poly-N-acetylglucosamine
PSMs – Phenol-soluble modulins

PVL – Panton-Valentine leucocidin
QACs – Quaternary ammonium compounds
QRDR – Quinolone resistance determining-region
qRT-PCR – Quantitative real time – polymerase chain reaction
RNA – Ribonucleic acid
RND – Resistance-nodulation-cell division superfamily
rRNA – Ribosomal ribonucleic acid
RRDR – Rifampicin resistance determining-region
SaPIs – *Staphylococcus aureus* pathogenicity islands
SCC*mec* – Staphylococcal cassette chromosome *mec*
SCENIHR – Scientific committee on emerging and newly identified health risks
SERPA – Serological proteome analysis
SFP – Staphylococcal food poisoning
SIET – *Staphylococcus intermedius* exfoliative toxin
SIG – *Staphylococcus intermedius* group
SLST – Single-locus sequence typing
SLV – Single locus variant
SMR – Small multidrug resistance family
spa – Staphylococcal protein A
SSTIs – Skin and soft tissue infections
ST – Sequence type
TSS – Toxic shock syndrome
TSST – Toxic shock syndrome toxin
UTIs – Urinary tract infections
VRSA – Vancomycin-resistant *Staphylococcus aureus*

1 Introduction

1.1 The taxonomy of staphylococci

Staphylococci are a group of Gram-positive bacteria that were first described in 1880 by Sir Alexander Ogston (Ogston, 1882). The *Staphylococcus* genus includes at least 60 species and subspecies, divided into 2 major groups: coagulase-positive and coagulase-negative staphylococci (CoPS and CoNS, respectively) (Lamers, Muthukrishnan, Castoe, Tafur, Cole & Parkinson, 2012). This division is based on the production of an enzyme, coagulase, which promotes blood clot formation (Blair, 1962). Staphylococci are part of the normal flora of animals and humans, however they have an opportunistic character (Fischetti, Novick, Ferreti, Portnoy & Rood, 2006). What turns staphylococci so interesting is their ability to live in perfect commensalism with their hosts and yet their ability to infect when the opportunity arises (Fischetti et al., 2006). Furthermore, staphylococci are not able to cause only one type of infection; on the contrary they can originate a variety of diseases that can range from a simple pustule to a life-threatening endocarditis (Fischetti et al., 2006). Erstwhile, only CoPS were believed to cause disease and CoNS were thought to be non-pathogenic (Blair, 1962). Yet in our days almost all *Staphylococcus* species are known to be able to cause infections (Fischetti et al., 2006).

To date seven species of CoPS group have been identified: *S. aureus*, *S. intermedius*, *S. schleiferi* subsp. *coagulans*, *S. hyicus*, *S. lutrae*, *S. delphini* and *S. pseudintermedius* (Sasaki, Tsubakishita, Tanaka, Sakusabe, Ohtsuka, Hirotaki, Kawakami, Fukata & Hiramatsu, 2010). Within these species, *S. aureus* is considered the most important staphylococcal species in human medicine due to its virulence and capacity to acquire/develop antimicrobial resistance (Fischetti et al., 2006) and *S. aureus* and *S. pseudintermedius* in veterinary medicine (Weese and van Duijkeren, 2010). Species identification is difficult when based on phenotypic methods, due to a lack of unique biochemical markers. Yet this discrimination is important because there are significant differences in species-specific antimicrobial breakpoints, like for example, for oxacillin (Sasaki et al., 2010). Thus, molecular identification of the most important species, especially *S. aureus*, is preferred to biochemical methods (Sasaki et al., 2010). Within the *S. intermedius* group (SIG), constituted by *S. intermedius*, *S. pseudintermedius* and *S. delphini*, discrimination can only be achieved through molecular identification. However, in veterinary medicine it is generally accepted that all strains belonging to the SIG from dogs are identified as *S. pseudintermedius*, unless genomic investigations prove that the strain belongs to another related species (van Duijkeren, Catry, Greko, Moreno, Pomba, Pyörälä, Ružaukas, Sanders, Threlfall, Torren-Edo & Törneke, 2011).

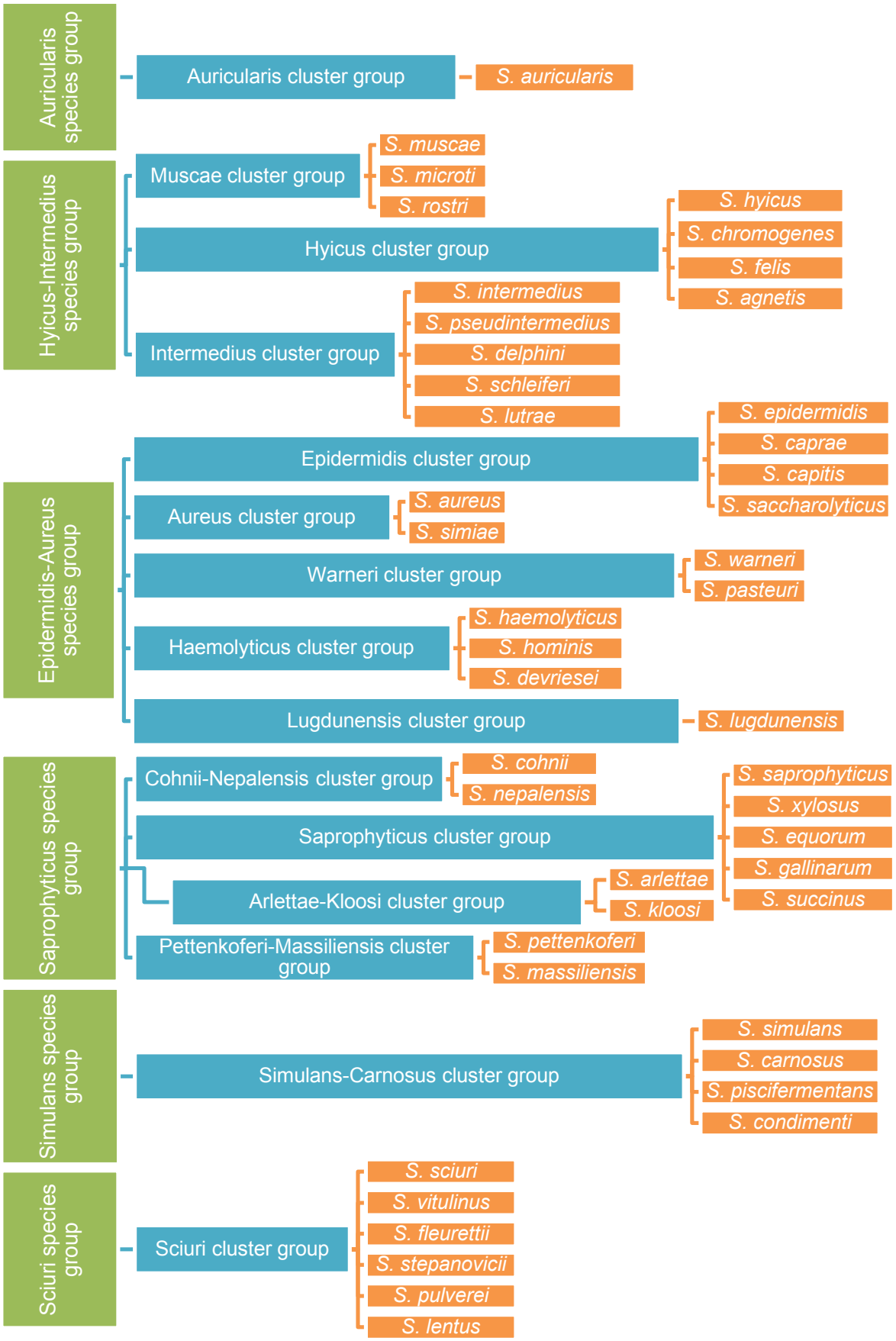
CoNS are normal inhabitants of skin and mucous membranes of animals and humans (Piette & Verschraegen, 2009). CoNS have long been disdained as culture contaminants, even in samples obtained from a normally sterile site by needle aspiration or surgery (Piette & Verschraegen, 2009). Only in 1958, Smith and colleagues published the first report on the potential pathogenicity of CoNS in patients with septicemia (Smith, Beals, Kingsbury & Has-

enclever, 1958). Yet, just in the 1970s, CoNS started to be recognized as etiologic agents of a wide variety of infections: bacteremia, central nervous system infection, endocarditis, urinary tract infection, surgical site infections, endophthalmitis, foreign body infection and many other infections (Piette & Verschraegen, 2009). CoNS are normally sub grouped in novobiocin-resistant species (*S. cohnii*, *S. saprophyticus*, *S. sciuri* and *S. xylosus*) and novobiocin-susceptible species (*S. auricularis*, *S. capitis*, *S. caprae*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis*, *S. pasteuri*, *S. saccharolyticus*, *S. schleiferi*, *S. simulans* and *S. warneri*) (von Eiff, Proctor & Peters, 2001). The best species identification method is by sequencing the *tuf* gene (Heikens, Fleer, Paauw, Florijn & Fluit, 2005). The most common CoNS species in human clinical samples are *S. epidermidis* (usually accounting for 50% of CoNS isolates), *S. haemolyticus* and *S. hominis* (Piette & Verschraegen, 2009), but several other species can also be found. In horses, cats, and dogs, CoNS are also the predominant species isolated from the skin (Lilenbaum et al., 1998; Schnellmann, Gerber, Rossano, Jaquier, Panchaud, Doherr, Thomann, Straub & Perreten, 2006; Schmidt, Williams, Pinchbeck, Corless, Shaw, McEwan, Dawson & Nuttall, 2014). One important feature of CoNS is their high rates of antimicrobials resistance: e.g. in humans it is generally assumed that approximately 80% of nosocomial isolates and 30-40% of isolates obtained from healthy carriers from the community are resistant to methicillin (Piette & Verschraegen, 2009). Meat and animal products are expected to be a significant reservoir of CoNS (Bhargava & Zhang, 2014).

S. agnetis and *S. schleiferi* are two species that include both CoPS and CoNS strains and are considered coagulase-variable staphylococci (Lamers et al., 2012). *S. schleiferi* is a well recognized human and veterinary pathogen and two subspecies have been identified: a coagulase-negative, *S. schleiferi* subsp. *schleiferi*, firstly isolated from humans; and a coagulase-positive subspecies, *S. schleiferi* subsp. *coagulans*, initially isolated from dogs with otitis externa (May, Hnilica, Frank, Jones & Bemis, 2005). More recently both subspecies were found in human and animal clinical samples (May et al., 2005).

Phylogenetic analysis suggests that staphylococcal species may be separated into six major staphylococcal species groups comprised of 15 refined cluster groups and this is shown in Figure 1 (Lamers et al., 2012). After this study was published, one novel species (and several subspecies) was identified from human clinical specimens *S. petrasii*, with phylogenetic relatedness, based on 16S ribosomal ribonucleic acid (rRNA), with the Haemolyticus cluster group species and *S. lugdunensis* (Pantůček, Švec, Dajcs, Machová, Černohlávková, Šedo, Gelbíčová, Mašlaňová, Doškař, Zdráhal, Růžičková & Sedláček, 2013). An overview of the staphylococcal species isolated in different hosts, food and environment is shown in Figure 2.

Figure 1. Staphylococcal species groups and clusters by phylogenetic analysis based on multilocus data (adapted from Lamers et al., 2012).



Fish
S. piscifermentans

Cheese
S. succinus
S. fleurettii

Milk
S. chromogenes
S. epidermidis
S. aureus

Meat
S. xylosus
S. carnosus
S. equorum

Figure 2. Wide distribution of staphylococcal species in animal and human hosts, food and environment.

Dogs
S. pseudintermedius
S. schleiferi
S. aureus

Otters
S. lutrae

Monkeys
S. simiae

Horses
S. equorum
S. aureus

Dolphins
S. delphini

Chicken
S. gallinarum
S. arlettae

Sheep
S. aureus
S. agneti

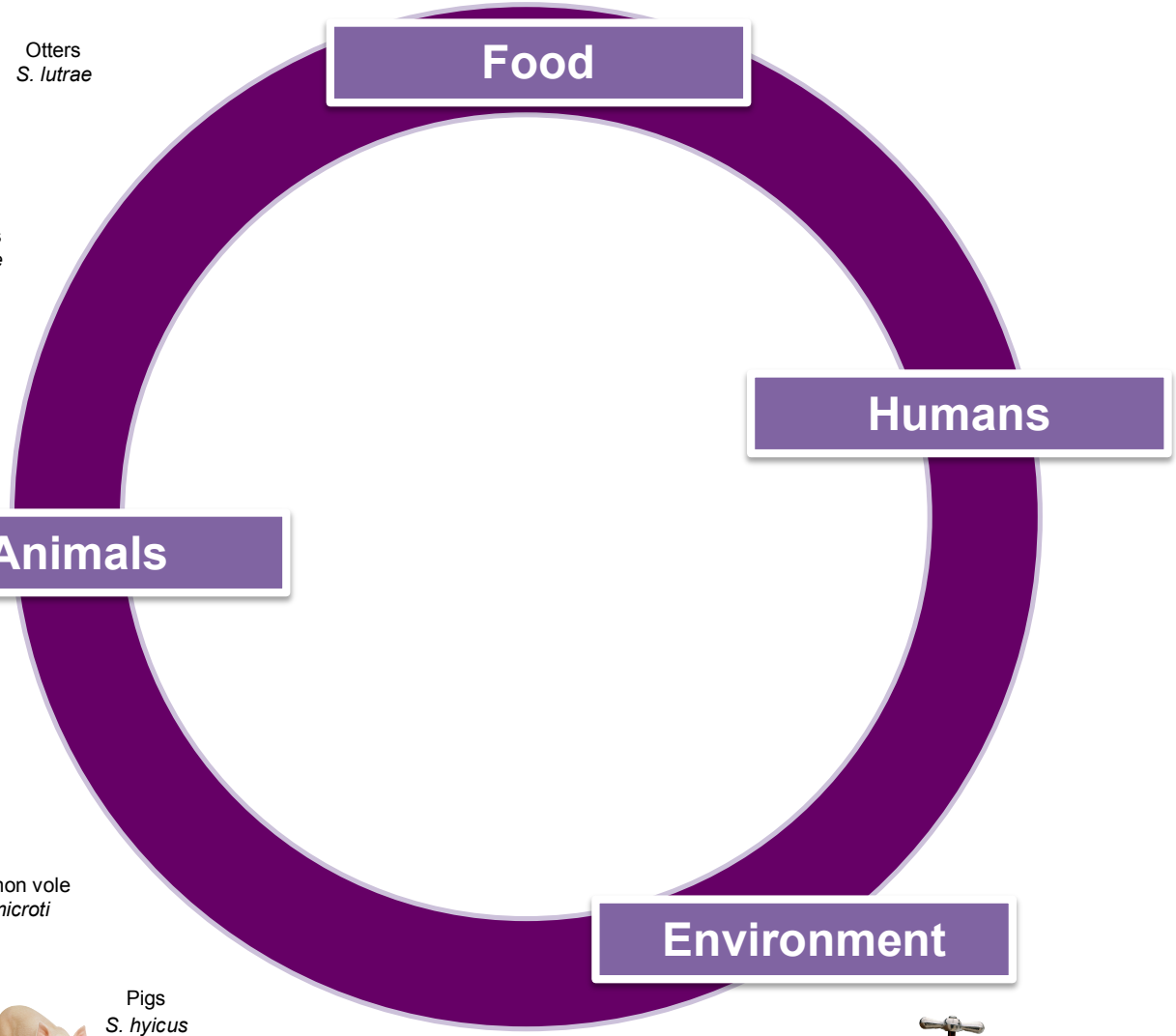
Cats
S. felis
S. pseudintermedius

Cows
S. aureus
S. chromogenes
S. epidermidis
S. simulans
S. warneri
S. devriesei

Common vole
S. microti

Goats
S. caprae
S. arlettae

Pigs
S. hyicus
S. rostri
S. aureus



Hospitals

S. aureus
S. epidermidis
S. haemolyticus
S. caprae

Community

S. aureus
S. epidermidis
S. haemolyticus
S. saprophyticus
S. lugdunensis
S. auricularis
S. capitis
S. hominis

Water
S. pasteuri
S. arlettae

Surfaces
S. xylosus

1.2 Staphylococci: commensals and pathogens

1.2.1 Staphylococci in humans

There are several staphylococcal species colonizing and infecting humans (Table 1), however *S. aureus* is regarded as the most important due to its pathogenic potential and ecology (Fischetti et al., 2006). *S. aureus* is a normal commensal of up to 50% of the anterior nares of healthy humans, either persistently or transiently (Frank, Feazel, Bessesen, Price, Janoff & Pace, 2010). Both healthy people and those with underlying illness are at risk for diverse infection types: abscesses, skin and soft tissue infections (SSTIs), endocarditis, osteomyelitis, meningitis, bacteraemia, urinary tract infections (UTIs), pneumonia and surgical site infections (Fischetti et al., 2006). Also, exotoxins produced by *S. aureus* can cause bullous impetigo, staphylococcal food poisoning (SFP), scalded skin syndrome, necrotizing pneumonia and toxic shock syndrome (Fischetti et al., 2006). Colonization of the nares is a risk factor for subsequent *S. aureus* infection, especially hospital-acquired bacteraemia (von Eiff, Becker, Machka, Stammer & Peters, 2001; Davis, Stewart, Crouch, Florez & Hospenthal, 2004).

Table 1. Examples of staphylococcal species identified in humans.

Species	Colonization /Infection	Type of infection
S. aureus	Both	Abscesses, SSTIs, endocarditis, osteomyelitis, meningitis, bacteraemia, UTIs, pneumonia, SFP, TSS and surgical site infections
S. auricularis	Both	Bacteraemia
S. capitis	Both	Bacteraemia, UTIs, wounds, prostatitis, endocarditis
S. caprae	Both	UTIs, SSTIs, bacteraemia, endocarditis and surgical site infections
S. cohnii	Both	Bacteraemia, wounds
S. condimenti	Infection	Bacteraemia
S. epidermidis	Both	Infections associated with implants, bacteraemia, UTIs, wounds, meningitis
S. equorum	Infection	Bacteraemia, meningitis, appendicitis
S. gallinarum	Infection	Bacteraemia
S. haemolyticus	Both	Infections associated with implants, bacteraemia, UTIs, wounds, meningitis, otitis, prostatitis
S. hominis	Both	Bacteraemia, wounds, abscesses, pneumonia
S. kloosi	Infection	Wounds
S. lugdunensis	Both	Endocarditis, bacteraemia
S. massiliensis	Both	Abscesses, wounds
S. nepalensis	Infection	UTIs
S. pasteuri	Both	UTIs, bacteraemia, SFP
S. petrasii	Infection	Otitis, bacteraemia

S. pettenkoferi	Both	Bacteraemia, osteomyelitis
S. pseudintermedius	Both	Dog bite wounds, rhino sinusitis, bacteraemia, SFP, infections associated with implants
S. saccharolyticus	Infection	Endocarditis, bacteraemia
S. saprophyticus	Both	UTIs, bacteraemia, endocarditis
S. schleiferi	Infection	Infections associated with implants
S. sciuri	Infection	Wounds, SSTIs, abscesses, peritonitis, endocarditis,
S. simulans	Both	UTIs, osteomyelitis, bacteraemia, endocarditis
S. succinus	Infection	Conjunctivitis, wounds
S. warneri	Both	Meningitis, wounds, endocarditis, bacteraemia
S. xylosus	Both	Bacteraemia, wounds, endocarditis, pneumonia

S. epidermidis, S. haemolyticus and *S. hominis* are the most frequently encountered CoNS species in human clinical samples (Piette & Verschraegen, 2009). *S. epidermidis* colonizes the skin and mucous membranes of humans and usually represents the major colonizer of these habitats (Vuong & Otto, 2002). As with the other staphylococci, *S. epidermidis* requires a predisposed host in order to cause infection (Vuong & Otto, 2002). An important virulence factor of *S. epidermidis* is the capacity to produce biofilm, allowing adhesion and permanence of bacteria on medical devices (catheters, prosthesis, etc.) and protection against the action of antimicrobials and the immune system (Piette & Verschraegen, 2009). *S. haemolyticus* is the second CoNS in its frequency of isolation from human blood cultures (Takeuchi, Watanabe, Baba, Yuzawa, Ito, Morimoto, Kuroda, Cui, Takahashi, Ankai, Baba, Fukui, Lee & Hiramatsu, 2005). *S. haemolyticus* is a normal commensal of the human skin microbiota, especially in areas where apocrine glands are found like the axillae, perineum and the inguinal area (Kloos & Bannerman, 1994). It is notorious for its multidrug resistance and historically early acquisition of resistance to methicillin and glycopeptide antimicrobials (Froggatt, Johnston, Galetto & Archer, 1989). This unusual presence of antimicrobial resistance genes is due to the extreme plasticity of its genome, given by the presence of many insertion sequence elements conferring frequent genomic rearrangements (Takeuchi et al., 2005).

S. saprophyticus is the second most frequent causative organism, after *Escherichia coli*, of uncomplicated UTI in women (Piette & Verschraegen, 2009). The vast majority of *S. saprophyticus* infections occur in young, sexually active women, but it can also cause UTI in males of all ages (Raz, Colodner & Kunin, 2005; Piette & Verschraegen, 2009). The urease of *S. saprophyticus* has been shown to contribute to its uropathogenicity as a major factor for invasiveness in bladder tissue (Hjelm & Lundell-Etherden, 1991). Furthermore, *S. saprophyticus* produces slime in urine and this slime production may be a risk factor for development of urinary stones, especially in concentrated urine in which the urea concentration is high (Hjelm & Lundell-Etherden, 1991). Interestingly, *S. saprophyticus* can be pathogenic in low numbers ($<10^5$ CFU/ml) (Rupp & Archer, 1994) and is probably often missed as causative

organism of UTIs, because if the bacterial counts are low, then the bacteriuria is considered non-significant (Piette & Verschraegen, 2009). The major reservoir of *S. saprophyticus* is the gastrointestinal tract (Piette & Verschraegen, 2009).

Several other CoNS are also associated with humans (Fischetti et al., 2006): for example *S. auricularis*, in conjunction with *S. capitis*, constitute the predominant Gram-positive, catalase-positive cocci inhabiting human ears (Kloos & Bannerman, 1994); *S. hominis* is a normal commensal of the human skin microbiota, especially in the axillae, perineum and inguinal areas of humans, areas where apocrine glands are found (Kloos & Bannerman, 1994); *S. lugdunensis* is a natural colonizer of humans, and is widely distributed over the body in small populations (Kloos & Bannerman, 1994); and *S. massiliensis* was firstly isolated from a human brain abscess (Masalma, Raoult & Roux, 2010) and later was proposed as a component of normal human skin microflora (Zong, 2012). CoNS infections in humans are becoming incredibly difficult to treat due to the high levels of antimicrobial resistance (Piette & Verschraegen, 2009).

Humans are not the natural host of *S. pseudintermedius*, but people exposed to animals, like owners and veterinary professionals, can become colonized/infected (Bond & Loeffler, 2012). The importance of this species as a zoonotic pathogen is less than that of *S. aureus* (Weese & van Duijkeren, 2010; van Duijkeren et al., 2011). However, although rare there are some reported cases of infection in humans, like for example, rhino sinusitis, bacteraemia and infections associated with implants (Stegmann, Burnens, Maranta & Perreten, 2010; Van Hoovels, Vankeerberghen, Boel, Van De Beenhouwer & Vaerenbergh, 2006; Chuang, Yang, Hsueh & Lee, 2010; Riegel, Jesel-Morel, Laventie, Boisset, Vandenesch & Prévost, 2011). In these cases, the origin of the strain was unknown but the person infected had contact with dogs (Chuang et al., 2010; Riegel et al., 2011), but as *S. pseudintermedius* was not searched in them, the zoonotic transmission was not proven (van Duijkeren et al., 2011).

1.2.2 Staphylococci in animals

Staphylococcus species have variable relevance in veterinary medicine, however, the most clinically relevant are the coagulase positive *S. aureus* and members of the *S. intermedius* group (SIG), particularly *S. pseudintermedius* (Weese and van Duijkeren, 2010).

1.2.2.1 Staphylococci in companion animals (dogs, cats and horses)

S. aureus has adapted in different animal species, including companion animals (Haenni, Targant, Forest, Sévin, Tapprest, Laugier, Madec, 2010; Weese and van Duijkeren, 2010). Wound infections, surgical site infections, pyoderma, otitis and UTIs are most commonly reported, but opportunistic infections at various other body sites can occur (Weese and van Duijkeren, 2010). In horses it can also cause sepsis, respiratory tract infections, and genital tract infections (Haenni et al., 2010).

S. pseudintermedius is a normal inhabitant of the skin and mucosa and can be isolated from the nares, mouth, pharynx, forehead, groin and anus of healthy dogs and cats (van Duijkeren et al., 2011). The anal region and the nose are colonized more frequently than other areas in healthy dogs (van Duijkeren et al., 2011). It is an opportunistic pathogen and a leading cause of pyoderma and otitis, infections of other body tissues and cavities, UTIs and post-operative wound infections in dogs and cats (Pomba, Couto & Moodley, 2010a; van Duijkeren et al., 2011). *S. pseudintermedius* is notoriously known for its ability to cause secondary infections in dogs with atopic dermatitis, an allergic skin disease, which leads to skin modifications that predispose for secondary infections (Nuttall, Uri & Halliwell, 2013).

S. schleiferi subsp. *coagulans* cause otitis and pyoderma in dogs, and rarely may be isolated from cats or birds (Davis, Cain, Brazil & Rankin, 2013). Prevalence from skin, nares, mouth, or perianal carriage in the absence of disease is low, typically ≤2%, but is higher among diseased pets (Davis et al., 2013). Of concern is the propensity for clinical isolates to be methicillin resistant, with many veterinary studies in the last decade reporting rates of 50% or higher (Davis et al., 2013).

CoNS are also important opportunistic pathogens in companion animals. *S. felis* is the most common staphylococci isolated from the skin surface of clinically normal cats (Lilenbaum et al., 1998), and has been associated with a variety of infections such as external otitis, cystitis, abscesses, wounds, and other skin infections (Igimi, Kawamura, Takahashi & Mitsuoka, 1989). *S. epidermidis* and *S. haemolyticus* can also colonize and cause infections in domestic mammals (Schmidt et al., 2014). *S. lentus*, *S. fleurettii*, and *S. vitulinus* can also be colonizers of dog's skin (Schmidt et al., 2014), while *S. equorum* comprises strains isolated from the skin of healthy horses (Schleifer, Kilpper-Bälz & Devriese, 1984).

1.2.2.2 Staphylococci in food-producing animals

Staphylococci can cause a variety of infections in food-producing animals, including bovine, ovine and caprine mastitis and exudative epidermitis in pigs (Fischetti et al., 2006). Bovine mastitis is the main cause of economic loss in milk production worldwide and *S. aureus* is the etiological agent more commonly associated with the disease and is normally related to subclinical or chronic infections (Pereira, Oliveira, Mesquita, Costa & Pereira, 2011). The role of the CoNS in bovine mastitis has come under increased scrutiny in recent years, and CoNS are among the most commonly isolated bacteria from heifers before and after parturition as well as from lactating cows (Aarestrup, Larsen & Jensen, 1999) In general, the most commonly isolated CoNS species from cases of bovine mastitis worldwide seems to be *S. chromogenes*, *S. epidermidis* and *S. simulans* (Aarestrup et al., 1999). *S. chromogenes* was actually the predominant CoNS species from bovine mastitis in Germany between 2003 and 2009 (Feβler, Billerbeck, Kadlec & Schwarz, 2010). Mastitis caused by CoNS is very mild and usually remains subclinical (Pyörälä & Taponen, 2009). However, the significance of

CoNS needs to be reconsidered, as in many countries they have become the predominant mastitis-causing agents (Pyörälä & Taponen, 2009).

In pigs *S. hyicus* is the main cause of exudative epidermitis (Tanabe, Sato, Sato, Watanabe, Hirano, Hirose, Kurokawa, Nakano, Saito & Maehara, 1996). Furthermore, *S. hyicus* has been isolated from animals with septic polyarthritis and bovine mastitis (Phillips, King & Kloos, 1980; Roberson, Fox, Hancock, Gay & Besser, 1996). Only a few cases of human infection have been published, namely, a wound infection after a donkey bite and bacteraemia in a farmer, in which the patients' close contact with piglets was the presumed source of infection (Osterlund & Nordlund, 1997; Casanova, Iselin, von Steiger, Droz & Sendi, 2011).

S. chromogenes is closely related to *S. hyicus* (Andresen, Ahrens, Daugaard & Bille-Hansen, 2005). *S. chromogenes* is part of the normal skin flora of pigs, cattle and poultry and has so far been considered non-pathogenic to pigs however, strains of *S. chromogenes* producing exfoliative toxin type B, ExhB, have been found as a cause of exudative epidermitis in pigs (Andresen et al., 2005). *S. aureus* can also cause exudative epidermitis in pigs (Pomba, Baptista, Couto, Loução & Hasman, 2010b). *S. rostri* is a recently described *Staphylococcus* species that is present in the nasal cavity of healthy pigs alone or in combination with *S. aureus* (Stegmann & Perreten, 2010). *S. rostri* can carry several antimicrobial resistance genes similar to *S. aureus* (Stegmann & Perreten, 2010).

S. caprae is the predominant species among the staphylococci recovered from mastitis-free goats' milk, as they colonize the healthy udder's skin (Devriese, Poutrel, Killper-Bälz & Schleifer, 1983; Bedidi-Madani, Kodjo, Villard & Richard, 1998). *S. gallinarum* is a staphylococcal species, whose natural host is birds: it was first described as a colonizer of chickens and a pheasant (Devriese et al., 1983), and more recently from a *Buteo buteo* (Sousa, Silva, Igrejas, Silva, Sargo, Alegria, Benito, Gómez, Lozano, Gómez-Sanz, Torres, Caniça & Poeta, 2014).

1.2.2.3 Staphylococci in other animals

Staphylococci can also colonize/infect several other species: *S. delphini* has been isolated from dolphins, minks, horses, cows and pigeons; *S. lutrae* isolated from otters; *S. intermedius* isolated from pigeons (Foster, Ross, Hutson & Collins, 1997; Ben Zakour, Beatson, van den Broek, Thoday & Fitzgerald, 2012); *S. muscae* was obtained from the body surfaces of flies caught in certain cowsheds but not on flies caught in human dwellings, stables, or piggeries and this bacterium was regarded as a transient rather than a resident on flies (Hájek, Ludwig, Schleifer, Springer, Zitzelsberger, Kroppenstedt & Kocur, 1992); *S. microti* was isolated from the common vole (*Microtus arvalis*) in 2010 (Nováková, Pantůcek, Hubálek, Falsen, Busse, Schumann & Sedlácek, 2010) and it was not reported ever since; *S. simiae* is a recently described species, isolated during an outbreak of diarrhoeal disease in squirrel monkeys in a zoo where staphylococcal strains with atypical features were isolated from both

ill and healthy animals (Pantůček, Sedláček, Petráš, Koukalová, Švec, Štětina, Vancanneyt, Chrastinová, Vokurková, Růžičková, Doškař, Swings & Hájek, 2005); *S. stepanovicii* has been isolated from wild small mammals (Hauschild, Stepanović & Zakrzewska-Czerwińska, 2010); and *S. warneri* resides in the skin epidermis of rainbow trout (*Oncorhynchus mykiss*) (Musharrafieh, Tacchi, Trujeque, LaPatra & Salinas, 2014).

1.2.2.4 Staphylococci in food products

Strains of the Simulans-Carnosus cluster group are commonly isolated from fermented foods (Tanasupawat, Hashimoto, Ezaki, Kozaki & Komagata, 1991). *S. carnosus* was originally isolated from fermenting sausages (Schleifer & Fischer, 1982). It has been shown that they exert positive effects on the formation of flavour and reddening reaction and therefore strains of this species are used as common components in starter cultures for the production of fermented sausage and cured ham (Hammes, Bosch & Wolf, 1995). *S. piscifermentans* was isolated in 1992 from fermented fish in Thailand (Tanasupawat, Hashimoto, Ezaki, Kozaki & Komagata, 1992). *S. condimenti*, can be isolated from fermented fish, shrimp sauces and soy sauce mash (Tanasupawat et al., 1991). *S. carnosus*, *S. condimenti*, *S. piscifermentans*, *S. equorum*, *S. succinus* and *S. xylosus* can be used as starter cultures for a variety of foods (Resch, Nagel & Hertel, 2008). *S. haemolyticus* and *S. hominis* can also be found in food of animal origin (Bhargavaa & Zhang, 2014). *S. succinus* subsp. *casei* has been obtained from the surface of ripened cheese (Nováková, Sedlácek, Pantůcek, Stetina, Svec & Petrás, 2006b).

S. saprophyticus has been isolated from rectal swabs taken from carcasses of cattle and pigs (Hedman, Ringertz, Lindstrom & Olsson, 1993). It was also found to contaminate 16% of various food samples in Sweden, with a high prevalence of 34% in samples of raw beef and pork (Hedman, Ringertz, Eriksson, Kvarnfors, Andersson, Bengtsson & Olsson, 1990). *S. saprophyticus* from food has been shown to carry *qac* genes (*smr* and *qacH*) and have been reported to produce enterotoxins (Heir, Sundheim & Holck, 1999).

S. warneri can be isolated from bovine milk and carry a high percentage of enterotoxin genes (de Freitas Guimarães, Nóbrega, Richini-Pereira, Marson, de Figueiredo Pantoja & Langoni, 2013). *S. warneri* and *S. pasteuri* isolated from milk often carry *qac*-efflux pumps (Bjorland, Steinum, Kvitle, Waage, Sunde & Heir, 2005). *S. auricularis* has been found in ready-to-eat fish and bovine milk, but usually do not carry any enterotoxin genes (de Freitas Guimarães et al., 2013; Sergelidis, Abrahim, Papadopoulos, Soultos, Martziou, Koulourida, Govaris, Pexara, Zdragas & Papa, 2014). *S. xylosus* is commonly present in raw meat and milk and is used as a starter culture for their fermentation (Dordet-Frisoni, Dorchies, De Araujo, Talo & Leroy, 2007).

1.2.2.5 Staphylococci in the environment

S. xylosus can persist in soils and on surfaces (Shale, Lues, Venter & Buys, 2006). One of the reasons they can persist is explained by its ability to form biofilms and the ability to adapt to different environments (Planchon, Gaillard-Martinie, Dordet-Frisoni, Bellon-Fontaine, Leroy, Labadie, Hebraud & Talon 2006; Dordet-Frisoni et al., 2007).

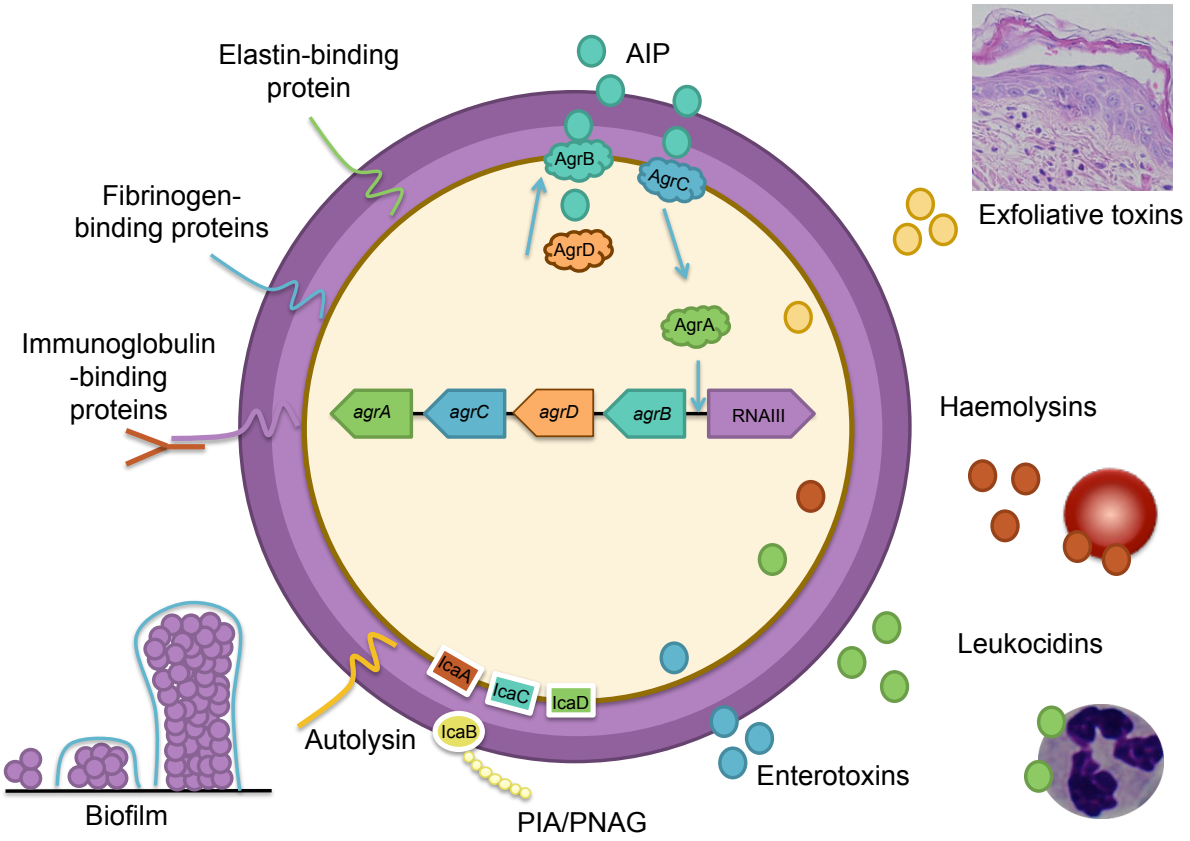
S. arlettae has two interesting properties: degrade textile azo dyes and promote plant growth and have been isolated from textile and tannery industrial effluents, respectively (Franciscon, Zille, Dias, Ragagnin, Durrant & Cavaco-Paulo, 2009; Sagar, Dwivedi, Yadav, Tripathi & Kaistha, 2012).

CoNS may also colonize water destined to human consumption, even when the quality standards for drinking water are fulfilled (Faria, Vaz-Moreira, Serapicos, Nunes & Manaia, 2009). The majority of the strains isolated from the drinking water distribution network belong to the species *S. pasteuri*, which has widespread distribution in food and environment, and may represent a relevant antimicrobial resistance reservoir, mainly in habitats with restrictive conditions (e.g. anaerobiosis) and reduced staphylococcal diversity (Faria et al., 2009).

1.3 Staphylococal virulence factors

Staphylococci are able to produce a wide range of virulence factors (Figure 3), enabling these species to infect the host (Fischetti et al., 2006). In this thesis, only the most relevant virulence factors will be reviewed.

Figure 3. Virulence factors produced by staphylococci.



1.3.1 Capsule

The capsule is composed of capsular polysaccharides and is a cell wall bacterial component that protects the bacteria from being phagocyted, enhancing staphylococcal virulence (Verdier, Durand, Bes, Taylor, Lina, Vandenesch, Fattom & Etienne, 2007). The capsule hampers the interaction between cell wall-bound C3b or immunoglobulin and receptors for these molecules on the phagocytic cell, resulting in bacterial evasion to the phagocytic uptake (O'Riordan & Lee, 2004). In the presence of antibodies specific for the capsule, C3b and antibody are deposited throughout the capsular matrix and on the bacterial surface, making them available for recognition by receptors on the phagocyte (Verbrugh, Peterson, Nguyen, Sisson & Kim, 1982). In the presence of specific capsular antibodies, mucoid strains are efficiently taken up by phagocytes and killed *in vitro* (Verbrugh et al., 1982). There are 11 capsular polysaccharide types but only two, types 5 and 8 (CP5 and CP8, respectively), are common in clinical isolates (Arbeit, Karakawa, Vann & Robbins, 1984; Verdier et al., 2007). Strains of serotypes 1 and 2, although rare, are heavily encapsulated (O'Riordan & Lee, 2004). Strains belonging to the remaining serotypes produce non-mucoid colonies on solid medium *in vitro*, and their colony morphology is indistinguishable from that of strains lacking a capsule (O'Riordan & Lee, 2004). Expression of *S. aureus* CP5 and CP8 *in vitro* is highly sensitive to various environmental signals and is probably influenced by the *in vivo* environment as well (O'Riordan & Lee, 2004). Capsule production *in vitro* is inhibited by yeast ex-

tract, alkaline growth conditions, and anaerobiosis (Stringfellow, Dassy, Lieb & Fournier, 1991) but enhanced by growth of the bacterium in milk (Sutra, Rainard & Poutrel, 1990), under iron limitation and on solid medium (Lee, Takeda, Livolsi & Paoletti, 1993) or in medium supplemented with up to 5% NaCl (Pohlmann-Dietze, Ulrich, Kiser, Doring, Lee, Fournier, Botzenhart & Wolz, 2000). *S. aureus* CP5 and CP8 are able to modulate abscess formation in an experimental rat model of intra-abdominal infection (Tzianabos, Wang & Lee, 2001). The capsule is also expressed in several models of infection, including endocarditis, subcutaneous infections and mastitis (Arbeit & Dunn, 1987; Lee et al., 1993; Hensen, Pavicić, Lohuis, de Hoog & Poutrel, 2000).

1.3.2 Enterotoxins

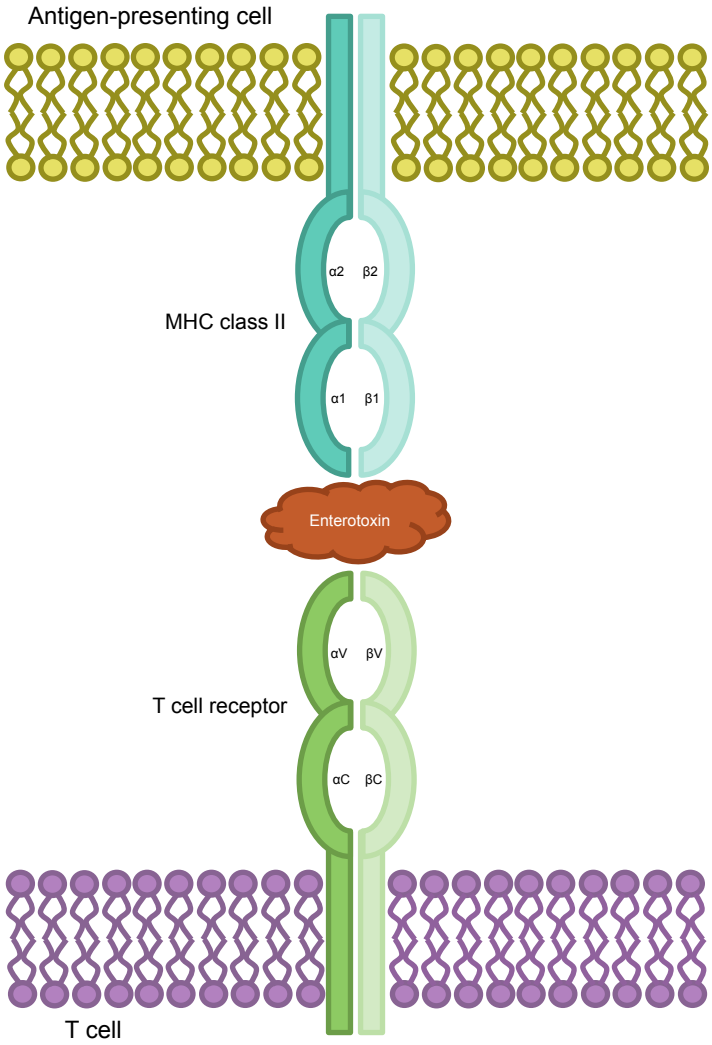
Enterotoxins were originally defined by their ability to cause staphylococcal food poisoning including emesis, and currently include the enterotoxins A, B, C, D, E, G, H, I, K, L, M, N, O, P, R, and T (Xu & McCormick, 2012; Omoe, Hu, Ono, Shimizu, Takahashi-Omoe, Nakane, Uchiyama, Shinagawa & Imanishi, 2013). The enterotoxin-like toxins, although both homologous and structurally similar to the enterotoxins, either do not induce emesis, or have not been formally demonstrated to induce emesis, and include the J, Q, S, U, V, and X (Xu & McCormick, 2012). Some enterotoxins have variants, for example SEC variants are *sec1*, *sec2*, *sec3*, $sec_{\text{MNCopeland}}$, sec_{4446}, sec_{bovine}, sec_{canine} and sec_{ovine} (Marr, Lyon, Roberson, Lupher, Davis & Bohach, 1993; Edwards, Deringer, Callantine, Deobald, Berger, Kapur, Stauffacher & Bohach, 1997; Fitzgerald, Monday, Foster, Bohach, Hartigan, Meaney & Smyth, 2001). SIG species genomes encode another enterotoxin called Se-int (Futagawa-Saito, Suzuki, Ohsawa, Ohshima, Sakurai, Ba-Thein & Fukuyasu, 2004). Enterotoxins are active in high nanogram to low microgram quantities (Evenson, Hinds, Bernstein & Bergdoll, 1988), and are resistant to heat treatment and low pH that easily destroy the bacteria that produce them, and to proteolytic enzymes, hence retaining their activity in the digestive tract after ingestion (Evenson et al., 1988). The actual mechanisms behind the emetic activity are poorly understood, but appear to involve the serotonin pathway (Hu, Zhu, Mori, Omoe, Okada, Wakabayashi, Kaneko, Shinagawa & Nakane, 2007). SFP is one of the most common foodborne diseases in the world following the ingestion of staphylococcal enterotoxins that are produced by enterotoxigenic strains of CoPS, mainly *S. aureus* (Jablonski & Bohach, 1997) and very occasionally by other staphylococci species such as *S. intermedius* (Genigeorgis, 1989; Khambaty, Bennett & Shah, 1994). However, CoNS can also produce enterotoxins (Crass & Bergdoll, 1986). Fourteen countries reported 346 outbreaks caused by staphylococcal toxins, representing 6% of all outbreaks reported in the European Union in 2012 (EFSA & ECDC, 2014). Food handlers carrying enterotoxin-producing *S. aureus* in their noses or on their hands are regarded as the main source of food contamination, via manual contact or through respiratory secretions (Argudín, Mendonza & Rodicio, 2010). Symptoms of

SFP have a rapid onset (2-8 h), and include nausea, violent vomiting, and abdominal cramping with or without diarrhoea (Murray, 2005). The disease is usually self-limiting and typically resolves within 24-48 h after onset but occasionally it can be severe enough to warrant hospitalization, particularly when infants, elderly or debilitated people are concerned (Murray, 2005).

Most genes coding for enterotoxins are located on mobile elements such as plasmids, bacteriophages or pathogenicity islands (Madhusoodanan, Seo, Remortel, Park, Hwang, Fox, Park, Deobald, Wang, Liu, Daugherty, Gill, Bohach & Gill, 2011; Suzuki, Kubota, Sato'o, Ono, Kato, Sadamasu, Kai & Kamata, 2015). One such element is the bovine pathogenicity island that encodes the three superantigens SEC_{bovine}, TSST-1 and SEL (Fitzgerald et al., 2001). The enterotoxin gene cluster (egc) comprises the five enterotoxin genes *seg*, *sei*, *sem*, *sen* and *seo* (Jarraud, Peyrat, Lim, Tristan, Bes, Mougel, Etienne, Vandenesch, Bonneville & Lina, 2001).

Staphylococcal enterotoxins and staphylococcal enterotoxin-like proteins can also be superantigens (Lina, Bohach, Nair, Hiramatsu, Jouvin-Marche & Mariuzza, 2004). Superantigens are secreted proteins that interact with antigen-presenting cells and T lymphocytes to induce cellular proliferation and high-level cytokine expression (Fast, Schlievert & Nelson, 1988; Kotb, 1995; Pinchuk, Beswick & Reyes, 2010). This process occurs as enterotoxins interact simultaneously with the major histocompatibility complex (MHC) class II molecule of the antigen-presenting cells and the Vβ domain of the lymphocyte T-cell receptor, forming trimolecular complexes (Figure 4) (Kotb, 1995). This interaction activates a much greater percentage of host T-cell repertoire than that induced by antigens presented in a normal manner (Xu & McCormick, 2012). This explains the massive cytokine expression and subsequent immunomodulation provided by these toxins (Xu & McCormick, 2012).

Figure 4. Interaction between enterotoxins and host cells (adapted from Pinchuk et al., 2010).



Some enterotoxins can also be involved in other staphylococcal syndromes besides food poisoning. Enterotoxins A to E, G and I can also be involved in staphylococcal toxic shock syndrome and staphylococcal scarlet fever (Schlievert, 1986; Yagoob, McClelland, Murray, Mostafa & Ahmad, 1990; Lina, Gillet, Vandenesch, Jones, Floret & Etienne, 1997; Jarraud, Cozon, Vandenesch, Bes, Etienne & Lina, 1999).

1.3.3 Leukocidins

Leukocidins are bi-component toxins encoded by several genetic loci (Fischetti et al., 2006). Leukocidins are two synergistically acting subunits consisting of S (slow) and F (fast) polypeptides, named after their electrophoretic mobility (Vandenesch, Lina & Henry, 2012). Depending on the combination of particular S and F polypetides, a toxin is formed with varying leukocytolytic, erythrocytolytic, and dermonecrotic properties (Vandenesch et al., 2012). In addition to neutrophils, the bi-component toxins are active against monocytes and macrophages (Vandenesch et al., 2012). They are pore-forming toxins and include (i) γ-haemolysin corresponding to two combinations of an S component (HlgA or HlgC) with an F component

(HlgB); (ii) the Panton-Valentine leukocidin (PVL), made of LukS-PV and LukF-PV; (iii) LukED; and (iv) LukGH, also known as LukAB (Vandenesch et al., 2012). PVL and γ-hemolysin are extremely potent, while LukAB/GH and lukED are active at concentrations 100-fold higher (Vandenesch et al., 2012). The bi-component toxins are largely considered to be secreted proteins (Vandenesch et al., 2012). LukGH/AB was identified both as a secreted protein and as one of the predominant surface protein of *S. aureus*, suggesting that this leukotoxin might be involved in targeting immune cells when they are in direct contact with the bacterium, such as during phagocytosis (Vandenesch et al., 2012). While γ-hemolysin, LukED and LukAB/GH are highly prevalent in *S. aureus* strains; PVL is produced only by 2 to 3% of the strains (Vandenesch et al., 2012). PVL is uncommonly found in methicillin-susceptible *S. aureus* (MSSA) and hospital-acquired (HA)-methicillin-resistant *S. aureus* (MRSA) isolates, but is associated with community-acquired MRSA (CA-MRSA) strains, which explains the frequency of primary skin infections and occasionally necrotizing pneumonia associated with these strains (Vandenesch, Naimi, Enright, Lina, Nimmo, Heffernan, Liassine, Bes, Greenland, Reverdy & Etienne, 2003). Various *lukS/lukF*-PV-transducing phages have been described, making PVL genes easily horizontally transmittable (Vandenesch et al., 2012).

S. pseudintermedius, *S. intermedius* and *S. delphini* may produce the bi-component leukotoxin Luk-I, made of LukF-I and LukS-I components (Prevost, Bouakham, Piemont & Monteil, 1995). Luk-I shows a strong leukotoxicity on various polymorphonuclear cells, but only a slight haemolytic activity on rabbit erythrocytes (Prevost et al., 1995).

1.3.4 Haemolysins

α-Haemolysin, like leukocidins, is also a pore-forming toxin (Vandenesch et al., 2012). Rabbit erythrocytes are highly sensitive to α-haemolysin-mediated lysis, but human erythrocytes are much less sensitive (Vandenesch et al., 2012). Furthermore, while human lymphocytes and monocytes are α-haemolysin-susceptible cells, granulocytes are highly resistant to lysis by this protein (Vandenesch et al., 2012).

β-Haemolysin does not form pores in the plasma cell membrane but instead is a neutral sphingomyelinase C hydrolysing sphingomyelin, which is a plasma membrane lipid (Vandenesch et al., 2012). β-haemolysin's enzymatic activity is required for its haemolytic activity (Vandenesch et al., 2012). *S. pseudintermedius* and *S. delphini* are also able to produce β-haemolysin (Ben Zakour et al., 2012).

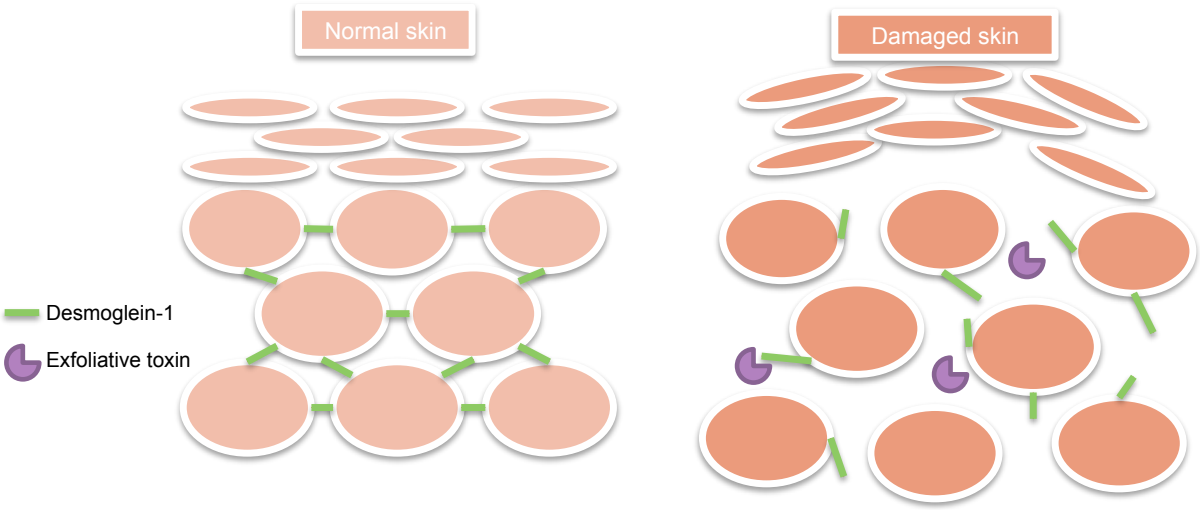
δ-Haemolysin is a small amphipathic (one hydrophobic and one hydrophilic side) peptide (26 AA) with a α-helix structure. Belongs to a family of small cytotoxic amphipathic peptides termed phenol-soluble modulines (PSMs) that were first identified in *S. epidermidis* and subsequently in *S. aureus* (Vandenesch et al., 2012). Two families of PSMs have been described based on their length: PSMα, including δ-haemolysin, PSMα1–4, and PSM-mec,

which are 20–26 aminoacids long; and PSMβ, including PSMβ1 and PSMβ2 that are 44 aminoacids long (Vandenesch et al., 2012). Three different mechanisms have been proposed to explain its haemolytic activity: δ-haemolysin could (i) bind to the cell surface and aggregate to form transmembrane pores; (ii) bind to the cell surface and disturb the membrane curvature, thus destabilizing the plasma membrane; or (iii) at high concentration, act as a detergent to solubilize the membrane (Vandenesch et al., 2012).

1.3.5 Exfoliative toxins

Staphylococcus species produce a variety of exfoliative toxins that induce different diseases in humans and animals (Terauchi, Sato, Endo, Aizawa & Maehara, 2003). In humans, staphylococcal scalded skin syndrome and bullous impertigo are caused by infection with *S. aureus* strains that produce exfoliative toxin A (ETA), B (ETB), or both (Kondo, Sakurai, Sarai & Futaki, 1975; Amagai, Yamaguchi, Hanakawa, Nishifuji, Sugai & Stanley, 2002). The gene encoding ETA is located on the chromosome whereas the gene encoding ETB is found on a large plasmid (Amagai et al., 2002). The two proteins specifically cleave desmoglein 1 as showed in Figure 5 (Amagai et al., 2002; Bukowski, Wladyka & Dubin, 2010).

Figure 5. Schematic representation of the desmoglein distribution in (A) healthy skin and (B) skin exposed to exfoliative toxin (adapted from Bukowski et al., 2010).



In *S. hyicus* four exfoliative toxins have been identified (Exfoliative Toxin Types A, B, C, and D). These toxins present in certain strains of *S. hyicus* enable the bacterium to cause a generalized skin disease named exudative epidermitis in pigs (Ahrens & Andresen, 2004). Exudative epidermitis is characterized by separation of the cells in the epidermis in the upper stratum spinosum, exfoliation of the skin, erythema, and serous exudation (Ahrens & Andresen, 2004). *S. pseudintermedius* strains can also produce exfoliative toxins named SIET (*Staphylococcus intermedius* exfoliative toxin) and EXI (Terauchi et al., 2003; Iyori, Futagawa-Saito, Hisatsune, Yamamoto, Sekiguchi, Ide, Son, Olivry, Sugai, Fukuyasu, Iwasaki & Nishifuji, 2011). EXI (also called ExpA), and not SIET, selectively digests canine desmoglein-

1, causes subcorneal clefts in canine epidermidis and may be involved in canine impetigo (Iyori et al., 2011). Recently, another exfoliative toxin gene, named SPETA, was identified in the *S. pseudintermedius* genome, but its function has not been demonstrated so far (Ben Zakour et al., 2012).

1.3.6 Other toxins

The toxic shock syndrome toxin (TSST) is one of the causative toxins of Toxic Shock Syndrome (TSS), producing high fever, headache, disorientation, vomiting, diarrhea and rashes. In the 80s, an increased number of studies reported more cases of TSS, which were soon associated with tampon use in young women. It was observed that those patients showed intra-vaginal colonization of *S. aureus* producing TSST (Torres, Kominsky, Perrin, Hobeika & Johnson, 2001). New animal variants were described subsequently, namely the ovine TSST and bovine TSST (Lee, Kreiswirth, Deringer, Projan, Eisner, Smith, Carlson, Novick & Schlievert, 1992).

1.3.7 Surface proteins

Surface proteins are usually anchored in the cell wall of staphylococci and interact with the bacterial environment (Navarre & Schneewind, 1999). Cell wall anchored proteins can be classified into four groups based on the presence of motifs shown in Table 2 (Foster, Geoghegan, Ganesh & Höök, 2014). The most prevalent group is the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) family, but other families include the NEAT motif family, the three-helical bundle family and the G5-E repeat family (Foster et al., 2014).

Table 2. Examples of staphylococcal cell wall anchored proteins.

Protein	Ligand	Staphylococcal species where it can be found
MSCRAMMs		
Clumping factor A (ClfA)	Fibrinogen Complement factor I	*S. aureus*
Clumping factor B (ClfB)	Fibrinogen Keratin 10 Loricrin	*S. aureus*
Staphylococcus pseudintermedius surface protein D (SpsD)	Fibrinogen Fibronectin Cytokeratin 10	*S. pseudintermedius*
Serine-aspartate repeat protein F (SdrF)	Collagen type I	*S. epidermidis*
NEAT motif family		
Iron-regulated surface protein A (IsdA)	Haemoglobin	*S. aureus*

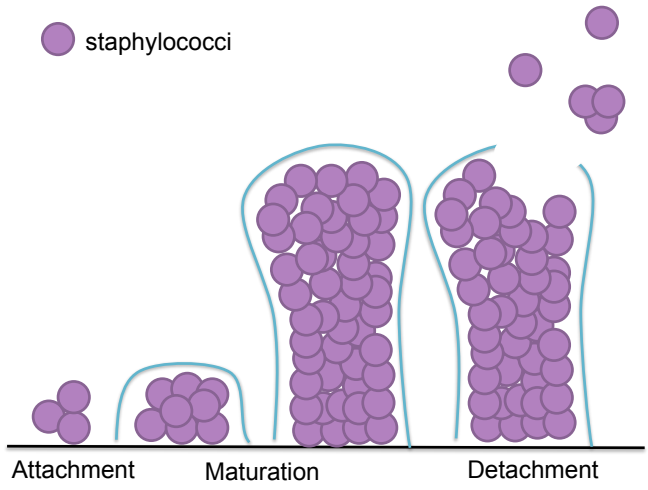
	Fibrinogen Fibronectin Cytokeratin 10 Loricrin	
Iron-regulated surface protein H (IsdH)	Haemoglobin	*S. aureus*
Three-helical bundle family		
Protein A	IgG Fc, IgM Fab	*S. aureus* *S. pseudintermedius* *S. hiycus*
G5-E repeat family		
S. aureus surface protein G (SasG)	Unknown ligand	*S. aureus*

These proteins fulfil a wide spectrum of functions, including binding to host tissues, interaction with adhesive matrix molecules, invasion, inflammation, immune evasion, biofilm formation, and binding of heme proteins for bacterial iron scavenging during infection (Foster et al., 2014). Interestingly, these proteins show functional plasticity, meaning several proteins have the same function and one protein has many functions (Foster et al., 2014). One consequence of this plasticity is that knocking one single protein might only partially affect the bacteria (Foster et al., 2014).

1.3.8 Biofilm

A biofilm is a bacterial community of cells attached to each other or to an interface that are embedded in a matrix of extracellular polymeric substances (Figure 6) (Donlan & Costerton, 2002). Biofilm formation is now recognized as an important virulence factor in several staphylococcal infections, including native valve endocarditis, otitis media, cystic fibrosis pneumonia and infections associated with implanted biomaterials (Jain & Agarwal, 2009). Biofilms have increased resistance to antimicrobials, and to immune system mechanisms of defence, like antimicrobial peptides and neutrophil phagocytosis (Costerton, Stewart & Greenberg, 1999).

Figure 6. Steps of staphylococcal biofilm formation.



1.3.8.1 Attachment

The first step of biofilm formation is attachment (Otto, 2008). Attachment to a biological surface, like the body, is promoted by MSCRAMMs that have the capacity to covalently bind to host matrix proteins (Patti, Allen, McGavin & Hook, 1994). Other proteins not covantly bound to the cell wall include the autolysin (Atl) that can also govern attachment (Otto, 2008). Staphylococcal autolysins can also bind to plastic surfaces (Heilman, Hussain, Peters & Gotz, 1997).

1.3.8.2 Maturation

The second step of biofilm formation is maturation (Otto, 2008). Maturation occurs either in a polysaccharide intercellular adhesion (PIA)-dependent (Jabbouri & Sadovskaya, 2010) or PIA-independent manner (Toledo-Arana, Merino, Vergara-Irigaray, Debarbouille, Penades & Lasa, 2005).

1.3.8.2.1 Maturation in a PIA-dependent manner

After attachment, staphylococci start to aggregate intercellularly (Otto, 2008). In this manner the main molecule responsible for intercellular adhesion is the polysaccharide intercellular adhesion (PIA), also called poly-N-acetylglucosamine (PNAG) (Mack, Fischer, Krokotsch, Leopold, Hartmann, Egge & Laufs, 1996). PIA/PNAG, together with other polymers such as teichoic acids and proteins, form the major part of the extracellular matrix of biofilm-forming staphylococci, called slime (Otto, 2008). PIA/PNAG biosynthesis is determined by the products of the intercellular adhesion (*ica*) locus (Heilman, Schweitzer, Gerke, Vanittanakom, Mack & Gotz, 1996). Usually this type of maturation is induced by osmotic stress, like for example when cultivating in broth medium with high concentrations of NaCl (O'Neill, Pozzi, Houston, Smyth, Humphreys, Robinson & O'Gara, 2007). This PIA/PNAG-mediated biofilm development is highly associated with MSSA and not with MRSA strains (O'Neill et al., 2007), although the *ica* operon is present in the majority of these last strains (O'Gara, 2007). The *ica* operon can even be transcribed and regulated in MRSA strains grown in medium containing NaCl or glucose, without the production of PIA/PNAG (O'Neill et al., 2007).

In *S. epidermidis*, one way of differentiating virulent from non-virulent strains is by detecting the *ica* operon (Frerbourg, Lefebvre, Baert & Lemeland, 2000). In fact, up to 80-90% of clinical isolates and only 5-30% of contaminant *S. epidermidis* have been shown to carry the *ica* gene (Ziebuhr, Heilmann, Götz, Meyer, Wilms, Straube & Hacker, 1997).

The *ica* operon has been found in other staphylococcal species like *S. pseudintermedius* (Singh, Walker, Rousseau & Weese, 2013), *S. haemolyticus* (Fredheim, Klingenberg, Rohde, Frankenberger, Gaustad, Flægstad & Sollid, 2009), *S. lugdunensis* (Frank & Patel, 2007), *S. caprae* (Allignet, Aubert, Dyke & El Solh, 2001), *S. saprophyticus* (Møretrø, Hermansen, Holck, Sidhu, Rudi & Langsrud, 2003), *S. caseolyticus* (Møretrø et al., 2003), *S. condimenti*

(Møretrø et al., 2003), *S. sciuri* (Møretrø et al., 2003), *S. simulans* (Møretrø et al., 2003), *S. capitis* (Møretrø et al., 2003) and *S. cohnii* (Møretrø et al., 2003).

1.3.8.2.2 Maturation in a PIA-independent manner

In cases of PIA-independent biofilm formation, adhesive proteins substitute for PIA (Otto, 2008). These may include the accumulation-associated protein (Aap), a protein responsible for 27% of the biofilm formation in strains isolated from prosthetic joint infections (Rohde, Burandt, Siemssen, Frommelt, Burdelski, Wurster, Scherpe, Davies, Harris, Horstkotte, Knobloch, Ragunath, Kaplan & Mack, 2007); the biofilm-associated protein (Bap), highly prevalent in *S. aureus* isolates from animals suffering from mastitis (Cucarella, Solano, Valle, Amorena, Lasa & Penades, 2001); an homolog protein named Bhp, found in human *S. epidermidis* strains and other CoNS (Zhang, Ren, Li, Wang, Fu, Yang, Qin, Miao, Wang, Chen, Shen, Chen, Yuan, Zhao, Qu, Danchin & Wen, 2003; Tormo, Knecht, Gotz, Lasa & Penades, 2005); and FnBPA and FnBPB, two fibronectin-binding proteins found in *S. aureus* strains (O'Neill, Pozzi, Houston, Humphreys, Robinson, Loughman, Foster & O'Gara, 2008). Teichoic acids may also have a role in the biofilm PIA-independent formation (Gross, Cramton, Gotz & Peschel, 2001). The FnBP-mediated biofilm development is usually induced by mild acid stress, like for example when isolates are grown in a medium with high concentrations of glucose (O'Neill et al., 2008). This biofilm phenotype is associated with MRSA strains (O'Neill et al., 2008). Biofilm formation by *S. haemolyticus* is also induced, when the organism is cultivated in medium with glucose, in a PIA-independent manner, however the components involved in the biofilm are not yet determined (Fredheim et al., 2009).

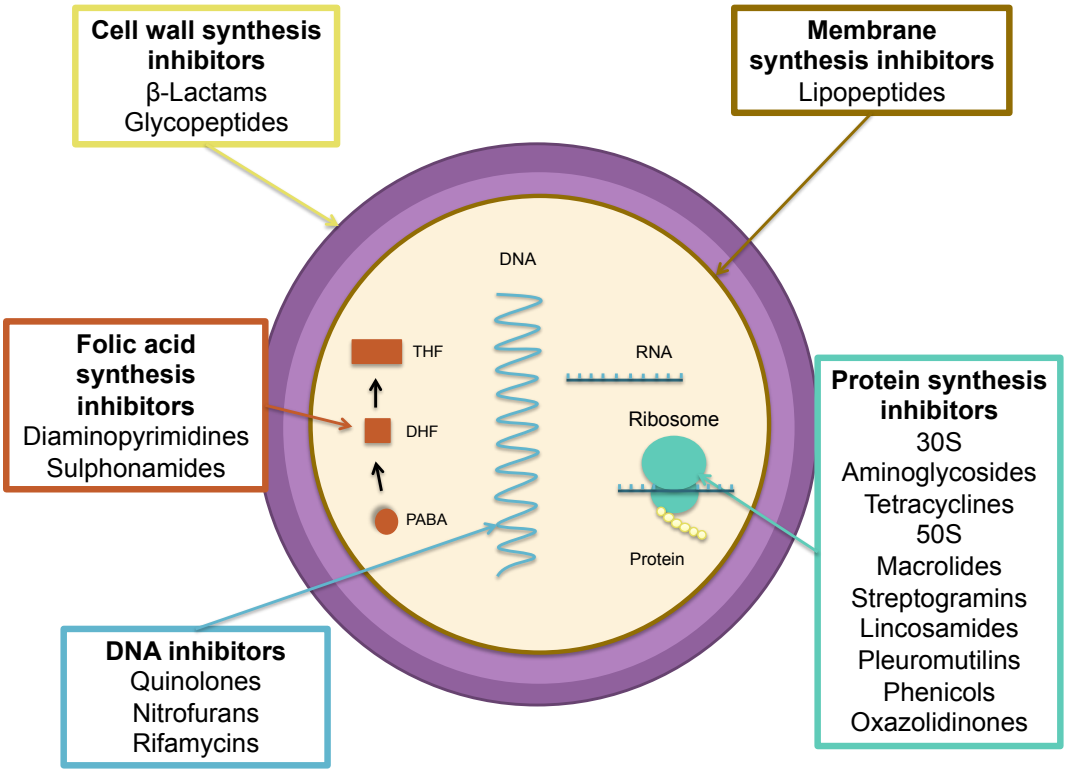
1.3.8.3 Detachment

Detachment is fundamental for the dissemination of bacteria to other colonization sites (Otto, 2008). Detachment can occur as single cells or large cell aggregates (Otto, 2008). Controlled detachment actually maintains a certain biofilm thickness and is controlled by the quorum-sensing system *agr* (Otto, 2008). The *agr* system regulates the expression of a class of peptides, named phenol-soluble modulins (PSMs), which when up-regulated lead to the detachment of cell clusters (Otto, 2008).

1.4 Treatment options against staphylococci: antimicrobials and biocides

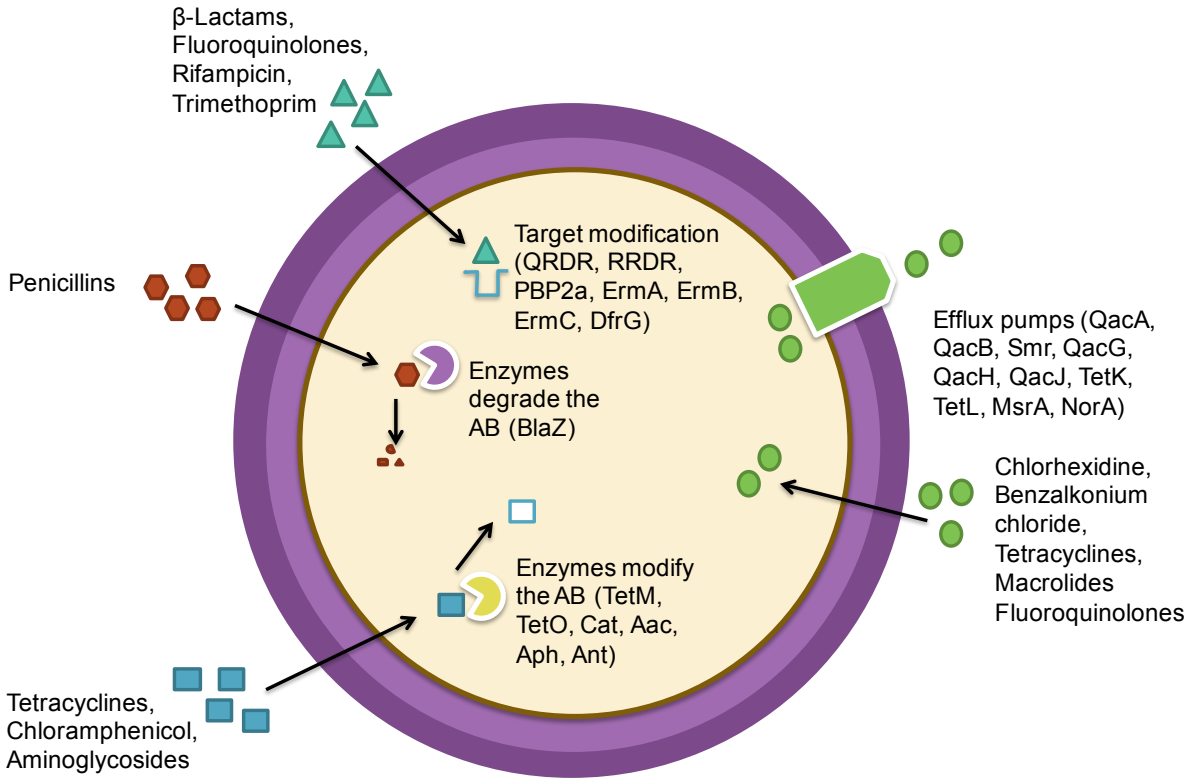
Antimicrobials are the first-line of choice for the treatment of staphylococcal infections. Antimicrobials are defined as naturally occurring or synthetic substances, which inhibit or destroy selective bacteria or other microorganisms, generally at low concentrations (Giguère, Prescott & Dowling, 2013). There are several classes of antimicrobials used to treat staphylococci infections both in human and veterinary medicine (Figure 7). A brief description of some of the antimicrobials used to treat staphylococcal infections is reviewed here.

Figure 7. Antimicrobial targets in staphylococci.



A large variety of antimicrobial resistance mechanisms have been described in staphylococci (Giguère et al., 2013). Antimicrobial resistance mechanisms can be classified into four major categories as shown in Figure 8.

Figure 8. Summary of the antimicrobial resistance mechanisms commonly found in staphylococci.



1.4.1 Cell-wall synthesis inhibitors

Cell-wall synthesis inhibitors are among the most selective antimicrobials, since they target a structure, the peptidoglycan, which only exists in prokaryotic and not in eukaryotes cells (Giguère et al., 2013). Antimicrobials affecting the cell wall synthesis include β-lactams (penicillins, cephalosporins, carbapenems and monobactams) and glycopeptides (Giguère et al., 2013). The peptidoglycan synthesis is done in four steps: 1) synthesis of the precursor in the cytoplasm; 2) precursor transport through the membrane; 3) deposition at the cell wall glycans; and 4) binding and maturation (McDermott, Walker & White, 2003). The β-lactams and glycopeptides operate in stages 3 and 4 of peptidoglycan synthesis (McDermott et al., 2003).

1.4.1.1 β-lactams

β-lactam antimicrobials were the first antimicrobials to be used in the clinical setting and are widely used due to their selectivity, versatility and low toxicity (Giguère et al., 2013). These antimicrobials reach bacterial killing by binding to penicillin-binding proteins (PBP) located in the cytoplasmic membrane that are involved in cell-wall assembly (Lowy, 1998; Gardam, 2000). By binding to these PBP, β-lactams consequently inhibit the crosslink of bacterial cell wall (Gardam, 2000). Penicillins are important antimicrobials for the treatment of infections in animals (Giguère et al., 2013). Penicillin G is the most commonly used antimicrobial in food-producing animals, especially ruminants, for the treatment of several bacterial infections (Giguère et al., 2013). In small animals, however, there are other formulations containing further penicillins in combination with β-lactamase inhibitors that are preferred (Giguère et al., 2013). Amoxicillin and ampicillin alone are also widely used in food-producing animals but they are susceptible to staphylococcal penicillinases (Giguère et al., 2013). Cloxacillin is antistaphylococcal, and is available as an intra-mammary formulation (Giguère et al., 2013). This penicillin is similar to methicillin and was developed to overcome the penicillinases or β-lactamases produced by staphylococci (Giguère et al., 2013). In small animals, amoxicillin-clavulanate (a β-lactamase inhibitor) is available to overcome these enzymes produced by staphylococci (Giguère et al., 2013). Ureidopenicillins, amidopenicillins and carboxypenicillins do not have activity against staphylococci (Giguère et al., 2013). Cephalosporins and carbapenems are resistant to staphylococcal penicillinases, however, carbapenems are not licensed for veterinary use and its administration in animals is poorly recommended (Giguère et al., 2013). Cephalosporins are divided into 5 generations, with 1st generation (e.g. cephalexin and cefazolin) and 5th generation (e.g. ceftobiprole and ceftaroline) being the most active against staphylococci (Kollef, 2009; Giguère et al., 2013). First generation cephalosporins are useful in the treatment of chronic staphylococcal pyoderma in dogs (Hillier, Lloyd, Weese, Blondeau, Boothe, Breitschwerdt, Guardabassi, Papich, Rankin, Turnidge & Sykes, 2014).

The initial mechanism of staphylococci resistance involved the production of penicillinases or β-lactamases, which hydrolyse the cyclic amide bond of the β-lactam ring (Gardam, 2000). Subsequently, after introduction of penicillinase-stable penicillins (like methicillin and oxacillin), a new mechanism of resistance developed and staphylococcal strains began producing a unique penicillin-binding protein, PBP2a or PBP2', which has a much lower affinity for β-lactam antimicrobials (including penicillins, cephalosporins, carbapenems) (Gardam, 2000; Weese & van Duijkeren, 2010). The first recognized gene encoding for PBP2a was *mecA*, which is part of a mobile genetic element, termed staphylococcal chromosome cassette *mec* (SCC*mec*) (Hiramatsu, Cui, Kuroda & Ito, 2001). More recently another gene, *mecC*, was identified in human and bovine *S. aureus* strains conferring resistance to β-lactam antimicrobials (García-Álvarez, Holden, Lindsay, Webb, Brown, Curran, Walpole, Brooks, Pickard, Teale, Parkhill, Bentley, Edwards, Girvan, Kearns, Pichon, Hill, Larsen, Skov, Peacock, Maskell & Holmes, 2011). This gene is located in a specific SCC*mec* type XI (García-Álvarez et al., 2011). The *mecA* and *mecC* genes have been detected in several staphylococcal species from humans and animals (García-Álvarez et al., 2011; Harrison et al., 2013; Harrison et al., 2014). *S. xylosus* harbour an allotype of the *mecC* gene, named *mecC1* (Harrison, Paterson, Holden, Morgan, Larsen, Petersen, Leroy, Vliegher, Perreten, Fox, Lam, Sampimon, Zadoks, Peacock, Parkhill & Holmes, 2013). Just like in *S. aureus mecC1* forms part of a class E *mec* complex (*mecI-mecR1-mecC1-blaZ*) located at the *orfX* locus as part of a likely SCC*mec* remnant, which also contains a number of other genes present on the type XI SCC*mec* (Harrison et al., 2013). Some strains harbouring the *mecC1* allotype may be cefoxitin- and oxacillin-susceptible due to inactivation of the resistance gene (Harrison et al., 2013). *S. saprophyticus* can carry another allotype of *mecC*, named *mecC2* (Matyszko, Schwarz & Hauschild, 2014).

1.4.1.2 Glycopeptides

Vancomycin, teicoplanin and avoparcin are glycopeptides with activity against Gram-positive bacteria, especially cocci (Guiguère et al., 2013). Vancomycin and teicoplanin are last-resort antimicrobials used for the treatment of MRSA infections in humans (Guiguère et al., 2013). Avoparcin was available for veterinary medicine, but since vancomycin-resistant enterococci started appearing in farm animals, its use was forbidden in Europe (Guiguère et al., 2013). These antimicrobials interact with the cell wall components, inhibiting the formation of the rigid backbone of the cell wall (Guiguère et al., 2013). *S. haemolyticus* was the first staphylococcal species that acquired resistance to the glycopeptide antimicrobials teicoplanin and vancomycin (Froggatt et al., 1989). Studies of vancomycin-resistant CoNS showed that they have altered crosslinks compared to susceptible strains (Billot-Klein, Gutmann, Bryant, Bell, Van Heijenoort, Grewal & Shlaes, 1996.). It has been suggested that these altered crosslinks may inhibit vancomycin binding to target peptides, but this hypothesis has yet to be

proven (Srinivasan, Dick & Perl, 2002). Vancomycin resistance in CoNS, namely in *S. haemolyticus* is likely multifactorial, but the exact mechanisms await elucidation (Srinivasan et al., 2002). More recently Van proteins, first isolated in enterococci, have been described in human *S. aureus* strains, including in Portugal (Melo-Cristino, Resina, Manuel, Lito & Ramirez, 2013). To date, seven types of resistance (VanA, -B, -C, -D, -E, -G, and -L) in enterococci have been described (Périchon & Courvalin, 2009). VanA-mediated resistance, the only to be described in *S. aureus*, is characterized by high levels of resistance to glycopeptides, vancomycin, and teicoplanin and is mediated by transposon Tn*1546* or closely related elements that are chromosomally- or plasmid-located (Périchon & Courvalin, 2009).

1.4.2 Membrane synthesis inhibitors

1.4.2.1 Lipopeptides

The bactericidal, cell membrane-targeting lipopeptide antimicrobial daptomycin is an important agent for the treatment of invasive *S. aureus* infections, especially since the emergence of vancomycin-resistant *S. aureus* (Bayer, Schneider & Sahl, 2013). This antimicrobial is only approved for the treatment of *S. aureus* skin and soft tissue infections, bacteraemia and right-sided endocarditis in human medicine (Bayer et al., 2013). However, there have been numerous recent reports of development of daptomycin-resistance during therapy with this agent (Bayer et al., 2013). The mechanism of resistance appears to be quite diverse, with strains often exhibiting progressive accumulation of single nucleotide polymorphisms in the multipeptide resistance factor gene (*mprF*) and the *yycFG* components of the *yycFGHI* operon (Bayer et al., 2013).

1.4.3 Deoxyribonucleic acid (DNA) inhibitors

1.4.3.1 Quinolones

The first quinolone approved for use in animals in 1988 was enrofloxacin (Giguère et al., 2013). Nowadays several others have been approved (Giguère et al., 2013). Quinolones are classified based on their biological activity (Andriole, 2005) as 1st generation quinolones with restricted antibacterial activity against Enterobacteriaceae (e.g. nalidixic acid, flumequine), 2nd generation quinolones with added spectrum against Gram-positive and improved activity against Gram-negative bacteria (e.g. for human purpose – norfloxacin, ofloxacin, ciprofloxacin, levofloxacin; veterinary purpose – danofloxacin, difloxacin, enrofloxacin, ibafloxacin, marbofloxacin, orbifloxacin [Table 3]), 3rd generation quinolones with greater potency against Gram-positive bacteria, particularly streptococci, and good activity against anaerobic bacteria (e.g. for human purpose – grepafloxacin, gatifloxacin, sparfloxacin, temafloxacin, tosufloxacin, and pazufloxacin; veterinary purpose – pradofloxacin) and 4th generation quinolones with potent activity against anaerobes and increased activity against pneumococci (e.g. for hu-

man purpose - trovafloxacin, clinafloxacin, sitafloxacin, moxifloxacin, and gemifloxacin) (Andriole, 2005).

Table 3. Fluoroquinolones approved for Veterinary Medicine in Europe (adapted from Giguère et al., 2013).

Fluoroquinolones	Species	Indications	Route of administration
Danofloxacin	Cattle, pigs, poultry, calves	Respiratory infections Mastitis (cattle) Gastroenteritis (calves)	Injectable
Difloxacin	Dogs, cats	SSTI, UTI	Oral
Enrofloxacin	Cattle, pigs, poultry, calves, dogs, cats	Respiratory infections Mastitis (cattle) Gastroenteritis (calves) SSTI, UTI (pets)	Oral, injectable
Ibofloxacin	Dogs, cats	SSTI, UTI, respiratory infections	Oral
Marbofloxacin	Cattle, pigs, calves, dogs, cats	Respiratory infections Mastitis (cattle, pigs) Gastroenteritis (calves) SSTI, UTI, otitis (pets)	Oral, injectable, topical
Orbifloxacin	Dogs, cats	SSTI, UTI, otitis	Oral, topical
Pradofloxacin	Dogs, cats	SSTI, UTI, periodontitis, respiratory infections, wounds	Oral

Resistance to fluoroquinolones occurs by target modification (topoisomerase IV and gyrase), decreased permeability, efflux and/or target protection (Hooper, 2002). More than one resistance mechanism can be present in one bacterial cell (Hooper, 2002). Amino acid changes in critical regions of the enzyme-DNA complex, named quinolone resistance–determining region (QRDR), reduce quinolone affinity for both its targets (topoisomerase IV and gyrase) (Lowy, 2003). The GrlA subunit of topoisomerase IV and the GyrA subunit in gyrase are the most common sites of resistance mutations, however topoisomerase IV mutations are the most critical, since they are the primary fluoroquinolone targets in staphylococci (Hooper, 2002). Single amino acid mutations are sometimes sufficient to confer clinical resistance, especially to 2nd and 3rd generation fluoroquinolones, but for the more active fluoroquinolones (4th generation) additional mutations are necessary (Lowy, 2003). Resistance mutations can accumulate in the QRDR sites, increasing the levels of resistance (Lowy, 2003) and it is very common that both targets, topoisomerase IV and gyrase, have mutations (Lowy, 2003). For example, moxifloxacin, a 4th generation fluoroquinolone with high activity against staphylococci, is active against ciprofloxacin-resistant *S. aureus in vitro* (Ince, Zhang & Hooper, 2003). Topoisomerase IV is the primary target of moxifloxacin in *S. aureus* and single mutations in the gene encoding this topoisomerase increases the MIC 4-8 times (Ince et al.,

2003). However, only a double mutation in topoisomerase IV and gyrase increases the MIC 125 times, enough to give resistance (Ince et al., 2003). An additional mechanism of resistance in *S. aureus* is induction of the Nor (NorA, NorB and NorC) efflux pumps (Lowy, 2003). Increased expression of these pumps in *S. aureus* can result in low-level quinolone resistance (Yoshida, Bogaki, Nakamura, Ubukata & Konno, 1990; Truong-Bolduc, Strahilevitz & Hooper, 2006; Ding, Onodera, Lee & Hooper, 2008). Overexpression of the NorA efflux pump, for example, causes a two-fold increase in the MIC of moxifloxacin in *S. aureus* (Ince et al., 2003).

As for *S. aureus*, ciprofloxacin resistance in CoNS is due to the combined presence of single mutations in each *grlA* and *gyrA* gene (Dubin, Fitzgibbon, Nahvi & John, 1999). For trovafloxacin resistance, a 4th generation fluoroquinolone, an additional mutation in the *grlA* gene is needed (Dubin et al., 1999). Some studies have shown a high frequency of fluoroquinolone resistance in *S. schleiferi* comparing to *S. pseudintermedius* (Intorre, Vanni, Di Bello, Pretti, Meucci, Tognetti, Soldani, Cardini & Jousson, 2007; Vanni, Tognetti, Pretti, Crema, Soldani, Meucci & Intorre, 2009). *S. schleiferi* and *S. pseudintermedius* fluoroquinolone-resistant isolates are usually resistant to 2nd and 3rd generation fluoroquinolones (enrofloxacin, levofloxacin, marbofloxacin, gatifloxacin) but susceptible to 4th generation fluoroquinolones (moxifloxacin, trovafloxacin) (Intorre et al., 2007; Vanni et al., 2009). Interestingly, for the same pattern of resistance, typically *S. pseudintermedius* shows double mutations in the *grlA* genes (positions 80 or 84) and *gyrA* (positions 84 or 88), while *S. schleiferi* only has a single alteration in *gyrA* (Intorre et al., 2007). However, until very recently none of these mutations were associated with resistance to 4th generation fluoroquinolones (Intorre et al., 2007). Yet, more recent studies on methicillin-resistant *S. pseudintermedius* (MRSP) have detected resistance to moxifloxacin (Couto, Pomba, Moodley & Guardabassi, 2011).

1.4.3.2 Nitrofurans

Nitrofurantoin is a nitrofuran with broad-spectrum activity against Gram-positive and Gram-negative bacteria (Maaland & Guardabassi, 2011). It is used for the treatment of UTIs in pets under the cascade (FAO/OIE/WHO 2008), since it is not approved for veterinary medicine however there is a lack of pharmacokinetic/pharmacodynamics studies in these species and there is a risk for adverse effects, including gastrointestinal irritation that may cause nausea and emesis in both dogs and cats (Maaland & Guardabassi, 2011). Yet, study results show that the use of nitrofurantoin might be indicated for the treatment of UTIs caused by staphylococci, including MRSP, which are otherwise difficult to treat using conventional veterinary antimicrobial products (Maaland & Guardabassi, 2011).

1.4.3.3 Rifamycins

Rifampicin (also called rifampin) acts by binding to the ß-subunit of the DNA-dependent ribonucleic acid (RNA) polymerase (Kadlec, van Duijkeren, Wagenaar & Schwarz, 2011). Rifampicin has a high degree of lipid solubility and so is effective against intracellular and extracellular pathogens (Giguère er al., 2013). Interestingly, rifampicin may enter neutrophils and macrophages to kill intracellular bacteria, without interfering with phagocytosis (Giguère er al., 2013). It is primarly used in veterinary medicine for the treatment of *Rhodococcus equi* infections in foals (Giguère er al., 2013). Rifampicin has been recently recommended for the treatment of MRSA and MRSP infections (Frank & Loeffler, 2012). However, resistance to rifampicin readily develops with monotherapy and should be used in combination with other antimicrobials to which the organism is also susceptible, such as clindamycin or cefalexin, to prevent development of resistance (Frank & Loeffler, 2012). Synergistic activity with erythromycin, clarithromycin and azithromycin has also been observed (Giguère er al., 2013).

In most bacteria, rifampicin resistance is mediated by mutations in the rifampicin resistance-determining region (RRDR) of the *rpoB* gene encoding the ß-subunit of RNA polymerase (Aubry-Damon, Soussy & Courvalin, 1998). Such mutations have been described in *S. aureus* and *S. pseudintermedius* (Aubry-Damon et al., 1998; Kadlec et al., 2011). Usually rifampicin-resistant isolates show mutations at one or two of the amino acid positions of RRDR (Kadlec et al., 2011).

1.4.4 Protein synthesis inhibitors

1.4.4.1 Aminoglycosides

Aminoglycosides are bactericidal antimicrobials with Gram-negative and Gram-positive activity, but they are not active under anaerobic condition and against anaerobes since the bacterial uptake is oxygen-dependent (Giguère et al., 2013). Several compounds within this class are approved for use in animals (Giguère et al., 2013). Aminoglycosides can cause varying degrees of ototoxicity and nephrotoxicity depending on the molecule, and for this reason this class is usually only used for the treatment of severe infections, such as septicaemias, digestive tract infections (e.g. neomycin for *Escherichia coli*), respiratory and urinary infections in many animal species (cattle, pigs, sheep, goats, horses, dogs and cats) (EMA, 2014). In the European Union the most frequently used aminoglycosides are neomycin and dihydrostreptomycin (EMA, 2014). Other substances from the group used in food producing species are: apramycin (approved for use in animals only), gentamicin, kanamycin, paromomycin, framycetin and streptomycin (EMA, 2014). Amikacin is an aminoglycoside that is not typically used in animals, however it is being used more frequently now, with the emergence of gentamicin-resistant MRSA and MRSP infections in dogs (Frank & Loeffler, 2012; EMA, 2014).

The most common mechanisms of resistance are the production of aminoglycoside modifying enzymes (Giguère et al., 2013). These resistance mechanisms are complex and differ between the different aminoglycoside molecules, and generally there is less cross-resistance when compared to other classes of antimicrobials (EMA, 2014). Enzymatic modification of aminoglycosides results in a chemical modification of the drug, with low affinity to bind to the ribosome (Mingeot-Leclercq, Glupczynski & Tulkens, 1999). Depending on the type of modifications they cause, these enzymes are called acetyltransferases (AAC), adenyltransferases (ANT) or phosphotransferases (APH) (Fluit, Visser & Schmitz, 2001). In staphylococci, usually the genes encoding these enzymes are present in plasmids or other mobile genetic elements (Giguère et al., 2013). Often there can be more than one aminoglycoside resistance gene in the same element (Giguère et al., 2013; McCarthy, Harrison, Stanczak-Mrozek, Leggett, Waller, Holmes, Lloyd, Lindsay & Loeffler, 2015). The bifunctional enzyme AAC(6′)-Ie-APH(2″) (also called AacA-AphD), conferring resistance to gentamicin, tobramycin and kanamycin, is the most common resistance mechanism found in *S. aureus* and CoNS isolated from humans and animals (Schmitz, Fluit, Gondolf, Beyrau, Lindenlauf, Verhoef, Heinz & Jones, 1999; Schnellmann et al., 2006). Resistance to neomycin, kanamycin, tobramycin and amikacin in staphylococci is mediated by an ANT(4′)-I (also called AadD) enzyme encoded by the *ant(4')-Ia* gene (Schmitz et al., 1999). The *str* gene, conferring resistance to streptomycin alone, has also been documented in CoNS from horses (Schnellmann et al., 2006). In *S. pseudintermedius*, the *aac(6')-Ie-aph(2")*, *ant(4')-Ia*, *ant(6')-Ie* and *aph(3')-IIIa* genes have been identified (Kadlec & Schwarz, 2012; Gold, Cohen & Lawhon, 2014; McCarthy et al., 2015). In MRSP, the *ant(6')-Ie* (also called *aadE*) and *aph(3')-IIIa* (also called *aphA*) genes are co-located on a Tn*5405*-like transposon (McCarthy et al., 2015). In 2010, a novel apramycin resistance gene, *apmA*, was detected in a bovine MRSA ST398 strain (Feβler, Kadlec & Schwarz, 2011). The *apmA* gene coded for a protein that was related only distantly to acetyltransferases involved in chloramphenicol or streptogramin A resistance (Feβler et al., 2011).

1.4.4.2 Tetracyclines

Tetracyclines, which were discovered in the 1940s, are a family of antimicrobials that inhibit protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site (Chopra & Roberts, 2001). Tetracyclines are broad-spectrum agents, exhibiting activity against a wide range of Gram-positive and Gram-negative bacteria, atypical organisms and protozoan parasites (Chopra & Roberts, 2001). The favourable antimicrobial properties of these agents and the absence of major adverse side effects has led to their extensive use in therapy of human and animal infections (Chopra & Roberts, 2001). Tetracyclines are first-line antimicrobials in food-producing animals, including aquaculture and honeybees, and are the class of antimicrobials with the highest use in veterinary medicine (Giguère et al., 2013). In

these animals, tetracyclines are used for a variety of infections, including respiratory infections (Giguère et al., 2013). In small animals, tetracyclines (e.g. doxycycline) are widely used to treat tick-born infections (Giguère et al., 2013). Minocycline was recently recommended for the treatment of MRSP infections in dogs, since these strains, although resistant to tetracycline, maintain their susceptibility to minocycline (Maaland, Guardabassi & Papich, 2014).

Resistance to tetracyclines can be mediated by several different mechanisms in staphylococci (Chopra & Roberts, 2001):

a) Efflux by reducing the intracellular concentration of the antimicrobial, conferring resistance to tetracycline but not minocycline (Chopra & Roberts, 2001). The genes *tet*(K) and *tet*(L), which confer such resistance, are generally found in small plasmids (Chopra & Roberts, 2001).

b) Ribosomal protection mediated by cytoplasmic proteins conferring resistance to tetracycline, doxycycline and minocycline (Chopra & Roberts, 2001). The genes *tet*(M) and *tet*(O) are the most studied and characterized in staphylococci (Chopra & Roberts, 2001).

c) Enzymatic inactivation of tetracycline, with only three genes have been identified so far: *tet*(X), *tet*(34) and *tet*(36) (Chopra & Roberts, 2001).

In CoNS from horses, *tet*(K) has been the most common tetracycline resistance gene detected but *tet*(M) has also been found (Schnellmann et al., 2006). So far, four different tetracycline resistance genes have been identified in *S. pseudintermedius* and SIG isolates: genes *tet*(K) and *tet*(L) coding for efflux pumps of the major facilitator superfamily, and the genes *tet*(M) and *tet*(O) coding for ribosome protective proteins (Kadlec & Schwarz, 2012).

1.4.4.3 Phenicols

Chloramphenicol is a broad-spectrum antimicrobial that has been used for several years in human and veterinary medicine (Schwarz, Kehrenberg, Doublet & Cloeckaert, 2006). Florfenicol, on the other hand, is licensed exclusively for veterinary medicine (Schwarz et al., 2006). Both antimicrobials have great activity against Gram-positive and Gram-negative bacteria (Schwarz et al., 2006). Chloramphenicol causes a number of adverse effects, including dose-unrelated irreversible aplastic anaemia and dose-related reversible bone-marrow suppression, and for this reason it is only limited to the therapy of a small number of life-threatening infections (Schwarz et al., 2006). Chloramphenicol was banned from use in any food-producing animals in the European Union (Schwarz et al., 2006). In small animals, chloramphenicol is used solely for ocular infections and otitis (Guiguère et al., 2013). Florfenicol does not cause adverse side effects like chloramphenicol and so it is licensed for the control of bacterial respiratory tract infections in cattle and pigs (Schwarz et al., 2006). These antimicrobials are bacteriostatic, inhibiting the protein synthesis by binding to the 50S ribosomal subunit (Schwarz et al., 2006). Resistance to chloramphenicol in staphylococci is usually

mediated by enzymatic inactivation through acetylation of the antimicrobial by chloramphenicol acetyltransferases (Cat) (Schwarz et al., 2006). The most commonly found in staphylococci are genes cat_{pC221} and cat_{pC223}, although cat_{pC194} has also been described (Schwarz et al., 2006). Small cat_{pC221}-carrying plasmids, ranging in size, have been identified in canine *S. pseudintermedius*, including MRSP (Kadlec & Schwarz, 2012). An efflux pump, FexA gives resistance to chloramphenicol and florfenicol (Kehrenberg & Schwarz, 2004). This protein was first described in a bovine *S. lentus* isolate (Kehrenberg & Schwarz, 2004), but it was then isolated from other staphylococcal species (Kehrenberg & Schwarz, 2006). Another mechanism of resistance to chloramphenicol and florfenicol is the one mediated by the *cfr* gene, encoding a methyltransferase that modifies 23S rRNA at A2503 (Long, Poehlsgaard, Kehrenberg, Schwarz & Vester, 2006). The *fexA* and *cfr* genes can be found in the same mobile genetic element (Kehrenberg & Schwarz, 2006). Recently there has been a new interest in the use of florfenicol as a second-line antimicrobial agent for the treatment of infections caused by MRSP (Maaland, Mo, Schwarz & Guardabassi, 2015). Yet, a novel *fexA* variant (*fexAv*) that confers only chloramphenicol resistance has already been described in *S. pseudintermedius*, with potential to reverse the naturally mutated positions and restore the florfenicol resistance phenotype (Gómez-Sanz, Kadlec, Feβler, Zarazaga, Torres & Schwarz, 2013).

1.4.4.4 Macrolides, Lincosamides and Streptogramins

Macrolide, lincosamide, and streptogramin (MLS) antimicrobials are widely used for the treatment of staphylococcal infections and are usually grouped together due to the overlapping binding sites and similar resistance mechanisms (Lina, Quaglia, Reverdy, Leclercq, Vandenesch & Etienne, 1999). Macrolides are classified according to the number of atoms comprising the lactone ring (Giguère et al., 2013). They have excellent activity against important bacterial pathogens of animals, especially food-producing animals (Table 4) (Giguère et al., 2013). Macrolides are able to accumulate within phagocytes (Giguère et al., 2013). Macrolides are usually bacteriostatic but they may be bactericidal at high concentrations (Giguère et al., 2013).

Table 4. Macrolide antimicrobials in veterinary medicine (adapted from Giguère et al., 2013).

Macrolide	Species	Indication	Route of administration
Gamithromycin	Cattle	Respiratory infections	Injectable
Spiramycin	Cattle, sheep, goats, poultry, pigs, dogs, cats	Respiratory infections Mastitis, metritis (cattle) Arthritis, metritis, enteritis, rhinitis (pigs) Stomatis (pets)	Oral, injectable

Tildipirosin	Cattle, pigs	Respiratory infections	Injectable
Tilmicosin	Cattle, pigs, poultry, rabbits	Respiratory infections	Oral, injectable
Tulathromycin	Cattle, sheep, goats, pigs	Respiratory infections Queratoconjunctivis	Injectable
Tylosin	Cattle, sheep, goats, pigs, poultry	Respiratory infections Mastitis, metritis (cattle) Arthritis, metritis, enteritis (pigs)	Oral, injectable
Tylvalosin	Pigs, poultry	Respiratory infections Gastroenteritis (pigs)	Oral

Lincosamides, comprising three different compounds used in veterinary medicine (lincomycin, clindamycin and pirlimycin) are active against Gram-positive bacteria, anaerobic bacteria and some mycoplasma (Giguère et al., 2013). The lincosamides, clindamycin and lincomycin, are used for the treatment of staphylococcal infections, mainly pyoderma and osteomyelitis in dogs and cats (Giguère et al., 2013). Lincomycin in association with neomycin can also be used as an intra-mammary administration for the treatment of mastitis (Giguère et al., 2013). Lincomycin associated to spectinomycin is approved for several animal species for the treatment of respiratory tract infections and lincomycin alone is frequently used in pigs to control dysentery and mycoplasma infections (Giguère et al., 2013).

Streptogramins are a group of natural (virginiamycin, pristinamycin) or semisynthetic (quinupristin/dalfopristin) cyclic peptides (Giguère et al., 2013). Streptogramins consist of two structurally unrelated molecules: group A streptogramins and group B streptogramins (Giguère et al., 2013). Virginiamycin is the only streptogramin approved for veterinary use (Giguère et al., 2013). These antimicrobials inhibit bacterial protein synthesis by binding irreversibly to the 50S ribosomal subunit (Giguère et al., 2013). Group A and B streptogramins bind to separate sites on the 50S ribosomal subunit: group A streptogramins bind first inducing a conformational change that increases affinity of the ribosome for group B streptogramins (Giguère et al., 2013).

Resistance to macrolides and lincosamides is prevalent among staphylococci, however, resistance against streptogramins, the two types A and B together, remains infrequent (Fluit et al., 2001). There are three basic mechanisms of resistance: i) target-site modification by methylation; ii) efflux of the antimicrobials; and iii) antimicrobial inactivation (Leclerq, 2002). Target modification alters a site in 23S rRNA common to the binding of macrolides, lincosamides and streptogramins B (MLS_B) (Fluit et al., 2001). This target modification confers cross-resistance to MLS_B antimicrobials and the genes encoding these methylases have been designated *erm* (erythromycin ribosome methylation) (Fluit et al., 2001). Streptogramins A-type are unaffected by these *erm* genes, and so susceptibility to compounds with

the two types of streptogramins is maintained (Fluit et al., 2001). Expression of MLS_B resistance in staphylococci can be constitutive or inducible and this is due to the regulatory region upstream of the structural gene for the methylase (Werckenthin, Schwarz & Westh, 1999). The Cfr rRNA methyltransferase, mentioned previously, also confers resistance to lincosamides and streptogramins A (Long et al., 2006).

Another mechanism of resistance is through antimicrobial modification: phosphotransferases (Mph) giving resistance to macrolides and lincosamide nucleotidyltransferases (Lnu) giving resistance to lincosamides (Leclerq, 2002). Genes *mph*(C) and *lnu*(A), encoding a phosphotransferase and a nucleotidyltransferase respectively, are quite uncommon in *S. aureus* but are frequent in CoNS (Leclerq, 2002). Other mechanisms include enzymes that hydrolyse streptogramins B [*vgb* genes (virginiamycin factor A hydrolases)] or modify streptogramins A by adding an acetyl group (acetyltransferases) [*vat*(A) (virginiamycin, factor A acetylation), *vat*(B), *vat*(C) genes] i (Roberts, Sutcliffe, Courvalin, Jensen, Rood & Seppala, 1999). Many of these genes are plasmid borne, and often these *vat*-related genes are downstream of other genes encoding resistance to streptogramins (Roberts et al., 1999).

The last resistance mechanism involves antimicrobial efflux through adenosine-triphosphate (ATP)-binding cassette (ABC) transporters (Leclerq, 2002). A specific efflux pump encoded by the gene *msr*(A) is present in staphylococci and effectively extrudes macrolides from the bacterial cell before they can bind to their target site on the ribosome (Lewis & Jorgensen, 2005). This mechanism of resistance does not create resistance to lincosamides, but only to 14-membered ring macrolides (e.g. erythromycin and clarithromycin), 15-membered ring macrolides or azalides (e.g., azithromycin, gamithromycin and tulathromycin), and group B streptogramins (e.g. quinupristin) (Lewis & Jorgensen, 2005). Resistance to streptogramins A and decreased susceptibility to lincosamides have also been attributed to ABC transporters encoded by the *vga* genes, described below (Gentry, McCloskey, Gwynn, Rittenhouse, Scangarella, Shawar & Holmes, 2008).

1.4.4.5 Pleuromutilins

There are two pleuromutilins used in veterinary medicine, tiamulin and valnemulin that have great activity against anaerobic bacteria and mycoplasma and They also against staphylococci (Giguère et al., 2013). Retapamulin is approved in Europe for topical use to treat impetigo, a highly contagious skin infection typically caused by *S. aureus* (Gentry et al., 2008). In Gram-positive bacteria, cross-resistance to pleuromutilins and streptogramins A and decreased susceptibility to lincosamides have been attributed to ABC transporters encoded by the *vga* genes (Gentry et al., 2008). To date, seven *vga* genes have been described in staphylococci: *vga*(A), *vga*(A)LC, *vga*(A)v, *vga*(B), *vga*(C) and *vga*(E) (Allignet, Loncle & El Sohl, 1992; Allignet & El Solh, 1997; Gentry et al., 2008; Kadlec & Schwarz, 2009; Haroche, Allignet, Buchrieser & El Solh, 2000; Schwendener & Perreten, 2011). These genes are very

common in staphylococci, especially in MRSA ST398 isolated from farm animals, and this is probably due to the use of antimicrobials like virginiamycin, tiamulin, valnemulin and lincomycin, that are administrated through medicated feed piggeries to prevent and treat bacterial infectious diseases (Schwendener & Perreten, 2011). Resistance to pleuromutilins can also be mediated by the, already mentioned, multidrug-resistance Cfr rRNA methyltransferase (Long et al., 2006).

1.4.4.6 Oxazolidininones

Oxazolidininones are a recently described class of antimicrobials (Giguère et al., 2013). Linezolid targets important functional centres of the ribosome of Gram-positive bacteria (Long et al., 2006). Linezolid was the first oxazolidininone to be approved in human medicine and it is mainly used for the treatment of MRSA and vancomycin-resistant *S. aureus* (VRSA) (Giguère et al., 2013). Treatment of MRSP infections with linezolid has been recommended (Papich, 2012), however this scenario represents a serious ethical dilemma, even when applying the cascade principle (Papich, 2012).

Resistance to oxazolidininones is usually conferred by the, already stated, multidrug-resistance Cfr rRNA methyltransferase (Long et al., 2006). The *cfr* gene was first described in 2000 in a bovine *S. sciuri* strain (Schwarz, Werckenthin & Kehrenberg, 2000). Since then it has been found in *S. simulans* from cows (Kehrenberg, Ojo & Schwarz, 2004) and in porcine *S. aureus* strains (Kehrenberg & Schwarz, 2006). The *cfr* gene is commonly detected in bacteria isolated from livestock, where the use of phenicols, lincosamides, and pleuromutilins may provide selective pressure and lead to maintenance of this gene in animal staphylococci (Tewhey, Gu, Kelesidis, Charlton, Bobenchik, Hindler, Schork & Humphries, 2014). Human *S. aureus* and *S. epidermidis* isolates can also harbour the *cfr* RNA methylase gene (Mendes, Deshpande, Castanheira, DiPersio, Saubolle & Jones, 2008; Tewhey et al., 2014).

Modification of the ribosome, commonly by mutation of the V domain of the 23S rRNA, is also associated with resistance in human clinical isolates of staphylococci (Tsiodras, Gold, Sakoulas, Eliopoulos, Wennersten, Venkataraman, Moellering, Ferraro, 2001; Mazzariol, Lo Cascio, Kocsis, Maccacaro, Fontana & Cornaglia, 2012; Tewhey et al., 2014). Multiple mutations in the rRNA and associated proteins have been descibed in linezolid-resistant isolates, underscoring the multifocal nature of resistance to linezolid in staphylococci (Tewhey et al., 2014).

1.4.5 Folic acid synthesis inhibitors

1.4.5.1 Sulphonamides

Sulphonamides interfere with the biosynthesis of folic acid in bacterial cells by competitively inhibiting the dihydropteroate synthase (DHPS) and consequently preventing the para-aminobenzoic acid from incorporating into the folic acid molecule (Lyon & Skurray, 1987).

Sulphonamides have a broad-spectrum of activity, however, resistance is very common, including in staphylococci (Giguère et al., 2013). At least two different mechanisms of bacterial resistance have been reported, but it is not known whether both are operative in staphylococci (Lyon & Skurray, 1987). One consists of an increased production of the para-aminobenzoic acid probably due to a chromosomal mutation (*sulA*); the other is due to a plasmid-encoded mechanism of resistance mediated by DHPS enzymes with much reduced affinity for the antimicrobial (Lyon & Skurray, 1987). So far, the molecular basis for sulphonamide resistance has not been identified in *S. pseudintermedius* (Kadlec & Schwarz, 2012).

1.4.5.2 Diaminopyrimidines

Trimethoprim is a diaminopyrimidine that interferes with folic acid synthesis by inhibiting the dihydrofolate reductase (Giguère et al., 2013). A synergistic and bactericidal effect is detected when a diaminopyrimidine is combined with a sulphonamide and formulations usually contain a combination of these antimicrobials (Giguère et al., 2013). Resistance to diaminopyrimidine is usually the result of synthesis of a resistant dihydrofolate reductase enzyme (Skold, 2001). The *dfr* genes encoding these enzymes are usually located on plasmids or transposons (Skold, 2001). Several *dfr* genes conferring resistance to trimethoprim have been described in staphylococci (Schnellmann et al., 2006). Usually the *dfr* genes found in CoPS and CoNS are different (Schnellmann et al., 2006). In CoNS *dfrA* and *dfrD* have been described (Schnellmann et al., 2006). In CoPS, the gene *dfrG* is the most common in *S. pseudintermedius* isolates, including MRSP (Kadlec & Schwarz, 2012); while the *dfrK* gene is the most common in *S. aureus* (Schnellmann et al., 2006).

1.4.6 Topical antimicrobials

1.4.6.1 Mupirocin

Mupirocin is a topical antimicrobial commonly used for topical treatment of canine bacterial skin infections caused by staphylococci (Werner & Russel, 1999). However, the use of this topical antimicrobial in veterinary medicine is controversial, since mupirocin is used for decolonization of persistent nasal MRSA carriage in humans (Coates, Bax & Coates, 2009). Mupirocin is an analogue of isoleucine that competitively binds to isoleucyl-tRNA synthetase, inhibiting protein synthesis (Farmer, Gilbart & Elson, 1992). Mupirocin-resistance is phenotypically divided into two groups, low-level (minimum inhibitory concentrations [MICs], 4 to 256 µg/ml) and high-level (MICs, ≥512 µg/ml) (Fluit et al., 2001). Low-level resistance to mupirocin in most cases is probably due to mutations in the host isoleucyl-tRNA synthetase, while isolates with high level resistance to mupirocin contain an additional biochemically distinct isoleucyl-tRNA synthetase, encoded by the *mupA* gene, that is less sensitive to inhibition by mupirocin (Gilbart, Perry & Slocombe, 1993). The *mupA* gene may be carried on transferable plasmids that vary in size in both *S. aureus* and CoNS isolates that are epidemi-

ologically unrelated (Farmer et al., 1992; Gilbart et al., 1993). One study in 2010 evaluated the *in vitro* susceptibility of staphylococci to mupirocin and found that 94% of the staphylococci, independent of the methicillin-resistant status, isolated from dogs with superficial pyoderma were susceptible to mupirocin (Fulham, Lemarie, Hosgood & Dick, 2010). Interestingly the odds of an isolate being resistant to mupirocin were nine times higher if the isolate was resistant to clindamycin than if the isolate was clindamycin-susceptible, suggesting cross-resistance (Fulham et al., 2010). In another study staphylococci from cats had statistically significant higher MICs for mupirocin than isolates from dogs (Loeffler, Baines, Toleman, Felmingham, Milsom, Edwards & Lloyd, 2008). Higher MICs of mupirocin were also significantly associated with infection isolates (Loeffler et al., 2008).

1.4.6.2 Fusidic acid

Fusidic acid is authorized for use in dogs and cats for the treatment of skin, ear or eye infections (Loeffler et al., 2008). Fusidic acid interacts with elongation factor G, preventing its release from the ribosome and thereby inhibiting bacterial protein synthesis (Turnidge & Collignon, 1999). Previously, mutations in elongation factor G encoding gene, *fusA,* were considered the primarily cause of resistance to fusidic acid (Turnidge & Collignon, 1999). However, in 2010, one study evaluated the fusidic acid resistance rates and resistance mechanisms among 4,167 *S. aureus* strains and 790 CoNS from North America and Australia and found an overall resistance prevalence of 1.7% (MIC ≥ 2μg/ml) (Castanheira, Watters, Bell, Turnidge & Jones, 2010). The CoNS strains carried *fusB* and *fusC* genes while *S. aureus* carried predominantly the *fusC* gene (Castanheira et al., 2010). Mutations in the *fusA* gene were also detected but only in a few *S. aureus* strains (Castanheira et al., 2010). The *fusD* gene is responsible for intrinsic fusidic acid resistance in *S. saprophyticus* strains (Skov, Frimodt-Moller & Espersen, 2001).

1.4.7 Biocides

Biocides are active chemical molecules, usually broad-spectrum in activity, that inactivate or kill microorganisms (Sheldon, 2005; SCENIHR, 2009). Biocides encompass chemicals with antiseptic, disinfectant, and/or preservative activity (Sheldon, 2005). Antiseptics are biocides that destroy or inhibit the growth of microorganisms in or on living tissue (e.g. health care personnel hand washes and surgical scrubs); and disinfectants are similar but are biocides that are used on inanimate objects or surfaces (Sheldon, 2005). Preservatives, on the other hand, are incorporated into pharmaceutical, cosmetic, or other types of products to prevent microbial contamination (Sheldon, 2005). The production, use and fate of biocides are well regulated by the European Committee (SCENIHR, 2009). Biocides can be used in a variety of settings, like health care, consumer products, food production, animal husbandry, foods of animal origin, and in the environment (Table 5) (SCENIHR, 2009).

The mechanism of action of biocides is poorly understood (Sheldon, 2005), but depends on the chemical characteristics of the biocide as well as the microorganism (Sheldon, 2005). Several other factors influence the biocide activity, like concentration, pH, duration of exposure and temperature (Sheldon, 2005).

Table 5. Biocides commonly used in human and veterinary medicine.

Biocide type	Examples	Use
Alcohols	Ethanol, Isopropanol	Antiseptic, disinfectant, preservative
Aldehydes	Formaldehyde, Glutaraldehyde	Disinfectant
Biguanides	Chlorhexidine (acetate or digluconate)	Antiseptic, disinfectant, preservative
Halogen-releasing agents	Sodium hypochlorite	Antiseptic, disinfectant
Peroxygens	Hydrogen peroxyde	Antiseptic, disinfectant
Phenylethers/Bisphenols	Triclosan	Antiseptic, preservative
Quaternary ammonium compounds (QACs)	Benzalkonium chloride, Cetrimide, CPC	Antiseptic, disinfectant, preservative

One product or formulation may have more than one biocide (SCENIHR, 2009). Usually the objective is synergy, meaning the combined action of the pooled biocides is greater than the sum of the activities of the biocides alone (SCENIHR, 2009). However, other effects can occur: additive, when the combined action of the added biocides is not greater than the sum of the individual biocides; and antagonistic, when the combined effect result is lower than the sum of the activities of the individual biocides (SCENIHR, 2009). Other components of a product or formulation may have an effect on the antimicrobial activity of the biocide (SCENIHR, 2009). For example, surfactants have an intrinsic antibacterial activity, and when used in combination with biocides, they increase the overall bactericidal activity (SCENIHR, 2009). Another example is Tris–EDTA, a chelating agent affecting permeability of the outer membrane in Gram-negative bacteria by removing Ca^{2+} and Mg^{2+} (Guardabassi, Ghibaudo & Damborg, 2009). This mechanism of action results in a synergistic effect when Tris–EDTA is administered together with other compounds, including antiseptics, most likely by enhancing their penetration into the bacterial cell (Guardabassi et al., 2009).

1.4.7.1 Biocide susceptibility

The objective of a biocide is to kill microorganisms very quickly, normally in a matter of minutes (SCENIHR, 2009). In this way, determination of MIC is not a good test to assess biocide susceptibility (Cerf, Carpentier & Sanders, 2010). Time is a fundamental factor to consider when testing biocide susceptibility (Cerf et al., 2010). Another important factor to pon-

der is temperature (Cerf et al., 2010). Biocides can be used in a large set of equipment, which are often at low temperatures (0ºC or less in refrigerators) or at high temperatures (100ºC in flow heat exchangers in the food industry) (Cerf et al., 2010). Furthermore, antiseptics used on the skin should do their activity at ± 37ºC (Cerf et al., 2010). The standardized methods to test for biocide susceptibility are based on the determination of the concentration that leads to a 5 logarithmic reduction in the number of microorganisms in a liquid suspension or deposited and dried on a carrier surface, in a given time at a given temperature, in the presence of interfering substances or not (Cerf et al., 2010). Interfering substances are proteins, carbohydrates and/or lipids, which bind to the biocides and reduce the concentration of molecules available to interact with the microorganism (Cerf et al., 2010). The European Committee for Standardization has 13 protocols available for the evaluation of biocides (CEN, 2006).

1.4.7.2 Inefficacy of biocides

The term biocide resistance is not a defined concept and is often misused (Cerf et al., 2010). A strain or a species is said to be “resistant” or “tolerant” or “non-susceptible” to a biocide if:

a) The time needed to achieve the 5 logarithmic reduction at a given biocide concentration is significantly longer than expected or;

b) The concentration needed to achieve the 5 logarithmic reduction for a given exposure time is significantly higher then normal.

This can be due to improper dilution of the in-use concentration of the biocide, to the presence of interference substances, to microorganism adhesion, to microorganism inactivation and/or to efflux pumps (Sheldon, 2005; Cerf et al., 2010). Improper dilution often occurs in places that should be but are not dry (e.g. floors or equipment) (Cerf et al., 2010). Insufficiently cleaned surfaces may contain organic matter that interferes with biocide activity (Cerf et al., 2010). Adherent microorganisms (in biofilms or not) are also harder to inactivate or kill than suspended bacteria and as a consequence the concentration needed to inactivate or kill a given fraction of an adherent population in a given time can be higher by more than 400-times than the one needed for cells in suspension (McDonnell & Russell, 1999). This is the reason why one of the available protocol tests of European Committee for Standardization uses a microbial population deposited and dried on a carrier surface (Cerf et al., 2010). Enzymatic inactivation of biocides has been described in bacteria, including staphylococci, notably to heavy metals (Ug & Ceylan, 2003; Cavaco, Hasman & Aarestrup, 2011). Efflux pumps decrease the intracellular concentration of biocides (Paulsen, Brown & Skurray, 1996). These efflux pumps are classified in five families according to their energy requirements and structure (Table 6) (Costa, Viveiros, Amaral & Couto, 2013):

a) Major facilitator superfamily (MFS);

b) Small multidrug resistance (SMR) family;

c) Multidrug and toxic compound extrusion (MATE) family;

d) Resistance-nodulation-cell division (RND) superfamily;

e) ATP-binding cassette (ABC) superfamily.

Several efflux pumps have been recognized in staphylococcal species (McDonnell & Russell, 1999; Sheldon, 2005). They can either be chromosomally- or plasmid-encoded (Costa et al., 2013). The Nor efflux-pumps, NorA, NorB and NorC, have been extensively characterized in *S. aureus* and one important characteristic is that they are capable of extruding fluoroquinolones, besides biocides (Yoshida et al., 1990; Truong-Bolduc et al., 2006; Ding et al., 2008). MepA is an efflux pump belonging to the MATE family, which is also capable of effluxing fluoroquinolones and also glycylcyclines, like tigecycline (Kaatz, McAleese & Seo, 2005; McAleese, Petersen, Ruzin, Dunman, Murphy, Projan & Bradford, 2005). Multidrug-efflux pumps of the MFS are capable of extruding, besides biocides, several antimicrobials namely virginiamycin, novobiocin, mupirocin, fusidic acid and fluoroquinolones by MdeA (Huang, O'Toole, Shen, Amrine-Madsen, Jiang, Lobo, Palmer, Voelker, Fan, Gwynn & McDevitt, 2004; Yamada, Shiota, Mizushima, Kuroda & Tsuchiya, 2006a) and oxazolidinones, phenicols, trimethoprim, erythromycin, kanamycin and fusidic acid by LmrS (Floyd, Smith, Kumar, Floyd & Varela, 2010). Both chromosomally encoded efflux systems SepA and SdrM only efflux low levels of biocides (Narui, Noguchi, Wakasugi & Sasatsu, 2002; Yamada, Hideka, Shiota, Kuroda & Tsuchiya, 2006b). Contrary to the chromosomally-encoded efflux pumps, the plasmid-mediated pumps have been detected in several staphylococcal species and are only able to efflux biocides (Costa et al., 2013). They are often reported in staphylococcal strains from food products and food-producing animals (Heir et al., 1999; Bjorland, Steinum, Kvitle, Waage, Sunde & Heir, 2005). But these pumps, especially QacA and QacB, are also commonly found in clinical samples (Alam, Kobayashi, Uehara & Watanabe, 2003; Correa, De Paulis, Predari, Sordelli & Jeric, 2008; Smith, Gemmel & Hunter, 2008). The genes encoding these pumps, *qacA* and *qacB* respectively, are often carried in the same mobile genetic element as the *blaZ* gene, encoding resistance to penicillin/ampicillin, in clinical and food-related staphylococci (Sidhu, Heir, Sørum & Holck, 2001; Sidhu, Heir, Leegaard, Wiger & Holck, 2002). Although Smr, QacG, QacH and QacJ have differences in their amino acid sequences, all these pumps share almost identical substrate specificities, extruding similar levels of benzalkonium chloride, ethidium bromide and cetyltrymethylammonium bromide (Costa et al., 2013).

Triclosan is often used to control MRSA outbreaks (Sheldon, 2005) and *in vitro* studies have showed that some *S. aureus* and *S. epidermidis* strains have higher MICs than others (Suller & Russell, 2000; Al-Doori, Morrison, Edwards & Gemmel, 2003; Schmid & Kaplan, 2004). A novel mechanism with high potential for horizontal transfer of decreased susceptibility to triclosan was described recently (Ciusa, Furi, Knight, Decorosi, Fondi, Raggi, Coelho, Aragones, Moce, Visa, Freitas, Baldassarri, Fani, Viti, Orefici, Martinez, Morrissey & Oggioni,

2012). The gene *sh-fabI* was suggested to have originated from *S. haemolyticus* core genome, transferred to *S. aureus* and from the *S. aureus* chromosome to plasmids (Ciusa et al., 2012). The *fabI* gene is the target of triclosan and the presence of the *sh-fabI* and the original *fabI* gene increases the target amount through heterologous target duplication (Ciusa et al., 2012).

Although these mechanisms are capable of interfering with biocide activity, their actual clinical significance remains unknown (Sheldon, 2005). Most of the times, in the literature we find "biocide resistance" associated with high MICs, yet the biocide in-use concentration is much higher than the MIC and so this is not truly resistance (Cerf et al., 2010). One study has evaluated the susceptibilities of MRSA isolates to seven biocides using the appropriate in-use concentrations and time of exposure and found some strains had minimum bactericidal concentrations (MBCs) to some biocides higher than user concentrations but failed to relate this inefficacy with the presence of efflux pumps (Narui, Takano, Noguchi & Sasatsu, 2007). However, some of these efflux pumps are able to secrete antimicrobials as well and the wide availability and common misuse of biocides can lead to the counter selection of staphylococci with these mechanisms (Sheldon, 2005). Likewise, the widespread use of antimicrobials can lead to the selection of bacteria carrying efflux-pumps and probably selecting for biocide tolerance (Sheldon, 2005).

1.5 Epidemiology of staphylococci

The epidemiology of infectious diseases relies on typing methods as tools for the characterization and discrimination of isolates based on their genotypic or phenotypic characteristics, which may be used to establish clonal relationships between strains and to trace the geographic dissemination of bacterial clones (Faria, Carriço, Oliveira, Ramirez & de Lencastre, 2008).

1.5.1 Single-locus typing methods

Single locus sequence typing (SLST) is used to determine the relationships among bacterial isolates based on the comparison of sequence variations in a single target gene (Sabat, Budimir, Nashev, Sá-Leão, van Dijl, Laurent, Grundmann & Friedrich, 2013). The most widely used method of the SLST group is the *S. aureus* protein A gene (*spa*)-typing, but other SLST are also useful (Sabat et al., 2013).

Table 6. Multidrug-resistant efflux pumps described in staphylococci (adapted from Costa et al., 2013).

Pump	Family	Substrate specificity	Staphylococcal species	Reference
Chromosomally encoded efflux pumps				
NorA	MFS	QACs, dyes and fluoroquinolones	*S. aureus*	Yoshida et al., 1990
NorB	MFS	QACs, dyes, fluoroquinolones and tetracyclines	*S. aureus*	Ding et al., 2008
NorC	MFS	Dyes, fluoroquinolones	*S. aureus*	Truong-Bolduc et al., 2006
MepA	MATE	QACs, dyes, fluoroquinolones, glycylcyclines	*S. aureus*	Kaatz et al., 2005
MdeA	MFS	QACs, dyes, fluoroquinolones, virginiamycin, novobiocin, mupirocin, fusidic acid	*S. aureus*	Huang et al., 2004; Yamada et al. 2006a
LmrS	MFS	QACs, dyes, oxazolidinones, phenicols, trimethoprim, erythromycin, kanamycin and fusidic acid	*S. aureus*	Floyd et al., 2010
SepA	Unknown	QACs, dyes, biguanidines	*S. aureus*	Narui et al., 2002
SdrM	MFS	Dyes, fluoroquinolones	*S. aureus*	Yamada et al., 2006b
Plasmid encoded efflux pumps				
QacA	MFS	QACs, dyes, biguanidines, diamidines	*S. aureus, S. epidermidis, S. haemolyticus, S. hominis, S. warneri*	Paulsen et al., 1996; Heir et al., 1999; Bjorland et al., 2005; Correa et al., 2008
QacB	MFS	QACs, dyes	*S. aureus, S. epidermidis, S. haemolyticus, S. hominis, S. warneri*	Paulsen et al., 1996; Heir et al., 1999; Bjorland et al., 2005; Correa et al., 2008
Smr or QacC	SMR	QACs, dyes	*S. aureus, S. caprae, S. cohnii, S. epidermidis, S. haemolyticus, S. hominis, S. pasteuri, S. saprophyticus, S. warneri*	Heir et al., 1999; Bjorland et al., 2005; Correa et al., 2008
QacG	SMR	QACs, dyes	*S. cohnii, S. haemolyticus, S. hominis, S. warneri*	Heir et al., 1999; Bjorland et al., 2005; Correa et al., 2008
QacH	SMR	QACs, dyes	*S. haemolyticus, S. saprophyticus*	Heir et al., 1998; Correa et al., 2008
QacJ	SMR	QACs, dyes	*S. aureus, S. delphini, S. intermedius, S. haemolyticus, S. hominis, S. simulans*	Bjorland et al., 2003; Bjorland et al., 2005; Correa et al., 2008

1.5.1.1 *spa* typing

In 1996, Frenay and colleagues developed a single-locus sequence typing method for *S. aureus* using the sequence of polymorphic X or short sequence repeat region of the *spa* gene as an alternative technique for the typing of *S. aureus* (Frenay, Bunschoten, Schouls, van Leewen, Vandenbroucke-Gauls, Verhoef & Mooi, 1996). The polymorphic X region consists of a variable number of 21 bp to 27 bp repeats and is located upstream of the region encoding the C-terminal cell wall attachment sequence (Schneewind, Model & Fischetti, 1992). The diversity of the region seems to arise from deletion and duplication of the repetitive units and also by point mutation (Brigido, Baradi, Bonjardin, Santos, Junqueira & Brentani, 1991). The existence of well-conserved regions flanking the X region coding sequence in *spa* gene allows the use of primers for polymerase chain reaction (PCR) amplification and direct sequence typing (Shopsin, Gomez, Montgomery, Smith, Waddington, Dodge, Bost, Riehman, Naidich & Kreiswirth, 1999). Moreover, the determination of *spa* types was simplified when appropriate software synchronized with an accompanying public website was developed (Harmsen, Claus, Witte, Rothgänger, Claus Turnwald & Vogel, 2003). A potential problem with *spa* typing is that it involves sequencing of only one small region of the chromosome, which is subject to recombination between unrelated clones (Cookson, Robinson, Monk, Murchan, Deplano, de Ryck, Struelens, Scheel, Fussing, Salmenlinna, Vuopio-Varkila, Cuny, Witte, Tassios, Legakis, van Leeuwen, van Belkum, Vindel, Garaizar, Haeggman, Olsson-Liljequist, Ransjo, Muller-Premru, Hryniewicz, Rossney, O'Connell, Short, Thomas, O'Hanlon, & Enright, 2007). The actual mutation rate in the *spa* region during long-term persistence has been reported to be one genetic change every 70 months (Kahl, Mellmann, Deiwick, Peters & Harmsen, 2005). This could result in isolates exhibiting the same *spa* type when they are unrelated by other methods (Cookson et al., 2007). Although *spa*-typing has a lower discriminatory ability than other molecular typing techniques (mainly pulsed-field gel electrophoresis [PFGE]) (Malachowa, Sabat, Gniadkowski, Krzyszton-Russjan, Empel, Miedzobrodzki, Kosowska-Shick, Appelbaum & Hryniewicz, 2005), its cost-effectiveness, ease of use, speed, excellent reproducibility, appropriate *in vivo* and *in vitro* stability, standardised international nomenclature, high-throughput by using a software, and full portability of data via the Ridom database, makes this method the currently most useful instrument for characterising *S. aureus* isolates at the local, national and international levels (Sabat et al., 2013).

The *spa* typing scheme for *S. pseudintermedius* was developed in 2009 and was tested against a collection of 31 MRSP isolates (Moodley, Stegger, Ben Zakour, Fitzgerald & Guardabassi, 2009). The results indicated there were two major MRSP clones: in Europe, clone sequence type (ST) 71-t02-III and in California, clone ST68-t06-V (Moodley et al., 2009). Like for MRSA, the developed *spa* typing method seemed promising for the easy and rapid typing of MRSP (Moodley et al., 2009). However, some issues were detected later.

Some MRSP and most methicillin-susceptible *S. pseudintermedius* (MSSP) strains could not be typed by this method (Perreten, Kadlec, Schwarz, Gronlund-Andersson, Greko, Moodley, Kania, Frank, Bemis, Franco, Iurescia, Battisti, Duim, Wagenaar, van Duijkeren, Weese, Fitzgerald, Rossan & Guardabassi, 2010; Ruscher, Lübke-Becker, Semmler, Wleklinski, Paasch, Soba, Stamm, Kopp, Wieler & Walther, 2010). The *spa* PCR products of non-typeable isolates could not be sequenced due to the presence of multiple bands that were found to be related to the presence of two adjacent *spa* genes (Perreten et al., 2010). For the purpose of overcoming this problem a nested PCR approach was described containing only the *spa* gene with the complete X-region (Perreten et al., 2010). However, another problem was that the same *spa* type could be found in unrelated STs and PFGE types (Perreten et al., 2010). This could be due to convergent evolution or genetic recombination (Perreten et al., 2010) and so care should be taken when interpreting variation in *spa* genes of *S. pseudintermedius* (Ruscher et al., 2010).

1.5.1.2 *agr* typing

The accessory gene regulator (*agr*) locus of *S. aureus* encodes a two-component signal transduction system that leads to down-regulation of surface proteins and up-regulation of secreted proteins during *in vitro* growth (Robinson, Monk, Cooper, Feil & Enright, 2005). Four auto-inducing peptide (AIP) variants of *agr* have been characterized in *S. aureus* (numbered I to IV) that generally induce *agr* activity within a group and inhibit *agr* activity between groups (Robinson et al., 2005). The inhibitory activity of *agr* groups may serve to isolate bacterial populations and facilitate the evolution of new strains or even species (Novick, 2003). This is based on the observation that a certain genetic background is usually represented by a given *agr* group; rarely is a given genetic background represented by multiple *agr* groups (Wright, Traber, Corrigan, Benson, Musser & Novick, 2005). Associations between *agr* group and certain strain characteristics may include resistance to glycopeptides (*agr* groups I and II) (Sakoulas, Eliopoulos, Moellering, Wennersten, Venkataraman, Novick & Gold, 2002; Verdier, Reverdy, Etienne, Lina, Bes & Vandenesch, 2004), isolation from toxic shock syndrome and from CA-MRSA disease (*agr* III) (Vandenesch et al., 2003), and isolation from staphylococcal scalded skin syndrome (*agr* IV) (Jarraud, Mougel, Thioulouse, Lina, Meugnier, Forey, Nesme, Etienne & Vandenesch, 2002). Epidemiological studies, however, generally conclude that *agr* groups have no obvious influence on strain colonization and competition dynamics in humans (van Leeuwen, van Nieuwenhuizen, Gijzen, Verbrugh & van Belkum, 2000; Robinson et al., 2005), questioning the proposal that *agr*-mediated bacterial interference is an important means of isolating bacterial populations (Robinson et al., 2005).

Like *S. aureus*, the *agrD* locus in *S. pseudintermedius* has four predicted AIP variants, I to IV, as shown in Table 7 (Sung, Chantler & Lloyd, 2006; Bannoehr, Ben Zakour, Waller,

Guardabassi, Thoday, van den Broek & Fitzgerald, 2007). The AIP variants found in *S. pseudintermedius* were also found in the other members of the SIG, suggesting the existence of a conserved *agr* quorum-sensing system (Bannoehr et al., 2007). In contrast to *S. aureus*, studies on *S. pseudintermedius* suggest that recombination has frequently contributed to the association of different *agr* alleles with strains of *S. pseudintermedius* of identical genotypes (Bannoehr et al., 2007). Also contrary to *S. aureus*, no association between *agr* types and host, clinical or geographic origin has been found in *S. pseudintermedius*, questioning what selective pressure drives *agr* diversification (Bannoehr et al., 2007).

Table 7. Amino acid sequences of the predicted *agrD*-encoded AIP identified in *Staphylococcus intermedius* group (adapted from Bannoehr et al., 2007).

agr type (AIP)	Amino acid sequence
I	RIPTSTGFF
II	RIPISTGFF
III	KIPTSTGFF
IV	KYPTSTGFF

1.5.1.3 *dru* typing

In 1991, a study conducted on the DNA sequence hypervariable region of the methicillin resistance determinant (*mec*), revealed a minimal direct repeat unit (*dru*) of 40 bp which was repeated 10 times within 500 bp (Ryffler, Bucher, Kayser & Berger-Bächi, 1991). These *dru* sequences are responsible for the length and polymorphisms of *mec* (Ryffler et al., 1991). Later on, Nishi and colleagues (1995) characterized this region in other *S. aureus* isolates as well as in methicillin-resistant coagulase-negative staphylococci (MRCoNS) and found that the region could have between 2 and 11 repeats in *S. aureus* and up to 16-18 repeats in *S. haemolyticus* (Nishi, Miyanohara, Nakajima, Kitajima, Yoshinaga, Maruyama & Miyata, 1995). Further studies using this epidemiological tool have concluded that the *mec*-associated *dru* typing may have potential for identifying and tracking specific subtypes of otherwise indistinguishable epidemic MRS (Nahvi, Fitzgibbon, John & Dubin, 2001; Goering, Morrison, Al-Doori, Edwards & Gemmell. 2008). Identical SCC*mec* types can have different *dru* types and the same *dru* type can sometimes be found in different SCC*mec* types (Bartels, Boye, Oliveira, Worning, Goering & Westh, 2013). The dt10a type has been proposed as the ancestor *dru* type for most MRSA lineages and the other *dru* types have evolved from this one (Bartels et al., 2013). MRCoNS have also been shown to carry dt10a (Bartels et al., 2013). MRSA *dru*-negative isolates have been found and these usually belong to the ST225 clone, which has suggested that the spread of this MRSA clone rather than several episodes of acquisition of an SCC*mec* lacking the *dru* region into the same genetic background has occurred (Bartels et al., 2013). For all these reasons, *dru* typing is not recommended as a

first line epidemiological typing method but might be informative for epidemiological subtyping and can add interesting information on the evolution of SCC*mec* (Bartels et al., 2013).

1.5.2 Multi-locus sequence typing (MLST)

MLST is a method of characterizing bacterial isolates on the basis of the sequences of ± 450 bp of internal fragments of seven housekeeping genes (Maiden, Bygraves, Feil, Morelli, Russell, Urwin, Zhang, Zhou, Zurth, Caugant, Feavers, Achtmann & Spratt, 1998). For each gene locus, unique sequences (alleles) are assigned arbitrary numbers and, based on the combination of identified alleles (i.e. the 'allelic profile'), the ST is determined (Sabat et al., 2013). MLST reveals slowly accumulating changes in conserved genes that reflect long-term evolutionary changes and can identify global spread of the relatively small number of successful clones (Gomes, Vinga, Zavolan & de Lencastre, 2005). MLST has become popular due to the development of large-scale sequencing methodologies, ease of data transfer and excellent comparability of results (Aires-de-Sousa, Boye, de Lencastre, Deplano, Enright, Etienne, Friedrich, Harmsen, Holmes, Huijsdens, Kearns, Mellmann, Meugnier, Rasheed, Spalburg, Strommenger, Struelens, Tenover, Thomas, Vogel, Westh, Xu & Witte, 2006), however, MLST is not appropriate for routine infection control due to its limited discriminatory power (Cai, Kong, Wang, Tong, Sintchenko, Zeng, & Gilbert, 2007).

In order to overcome the difficulty of comparing PFGE results and the genetic relatedness of the clones of MRSA described by different laboratories, Enright and colleagues (2000) developed a multilocus sequence typing for MRSA. Out of the fourteen housekeeping gene fragments sequenced, the seven housekeeping gene fragments that provided the greatest number of alleles were chosen for use in the MLST scheme and included: carbamate kinase gene (*arcC*), shikimate dehydrogenase gene (*aroE*), glycerol kinase (*glpF*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*) and acetyl coenzyme A acetyltransferase (*yqiL*) (Enright, Day, Davies, Peacock & Spratt. 2000).

The first MLST scheme described for *S. pseudintermedius* included the following 5 housekeeping genes: 16S rRNA (coding gene subunit ribosomal 16S rRNA), *cpn60* (chaperonin 60), *tuf* (elongation factor Tu), *pta* (acetyl phosphate) and *agrD* (accessory gene regulatory) (Bannoehr et al., 2007). However, this scheme was poorly discriminatory and was restructured to also include seven loci: *purA* (adenylosuccinate synthetase), *fdh* (formate dehydrogenase), *ack* (acetate kinase), *sar* (sodium sulfate co-transporter) and the already described above *pta*, *cpn60* and *tuf* genes (Solyman, Black, Duim, Perreten, van Duijkeren, Wagenaar, Eberlein, Sadeghi, Videla, Bemis & Kania, 2013). As for *S. aureus*, there is a worldwide database for MLST *S. pseudintermedius* (http://pubmlst.org/spseudintermedius) easily accessible.

Three different MLST schemes were published for *S. epidermidis* (Wang, Noble, Kreiswirth, Eisner, McClements, Jansen & Anderson, 2003; Wisplinghoff, Rosato, Enright, Noto, Craig &

Archer, 2003; Thomas, Vargas, Miragaia, Peacock, Archer & Enright, 2007). The first two described *S. epidermidis* MLST schemes were largely based on the scheme for *S. aureus* (Enright et al., 2000). By the use of different isolate collections, neither scheme was able to discriminate more than two CC (Thomas et al., 2007). A third scheme, assessed the ability of the 14 different loci used in these previous schemes (plus one scheme that was not published) to discriminate between isolates of *S. epidermidis* and determined an MLST scheme, which was not as discriminatory as those developed for other species, including *S. aureus* (Enright et al., 2000), but was improved enough to provide the best discrimination of the MLST formats available at the time for the typing of *S. epidermidis* strains (Thomas et al., 2007).

In the MLST websites there is an algorithm available, denominated BURST (Based upon related sequence type), that allows the grouping of related in STs into clonal complexes (CC) (Enright, Robinson, Randle, Feil, Grundmann & Spratt, 2002). STs are considered related if at least five of the seven loci are equal (Enright et al., 2002).

1.5.3 Pulsed-field gel electrophoresis (PFGE)

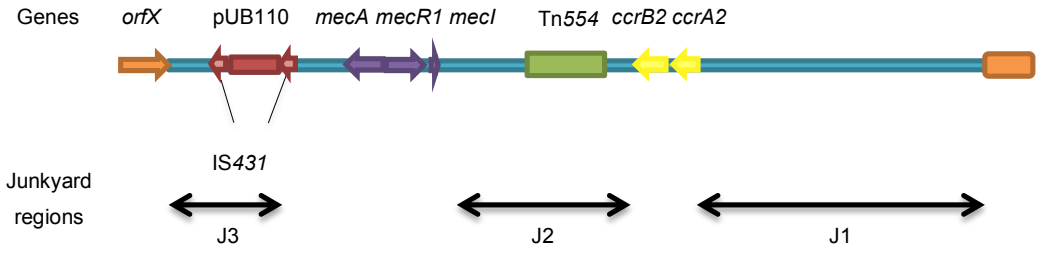
PFGE separates DNA under conditions of alternating polarity allowing for the resolution of DNA fragments nearly 20-times larger than those separated by traditional agarose gel electrophoresis (Leonard & Markey, 2008). PFGE is used in combination with restriction enzymes to give a DNA fingerprint of the bacterial genome (Tenover, Arbeit, Goering, Mickelsen, Murray, Persing & Swaminathan, 1995). The DNA restriction patterns of the isolates are then compared with one another to determine their relatedness (Tenover et al., 1995). The main advantage of this technique is that it provides great discrimination among strains and is useful in the investigation of outbreaks, by allowing differentiation of unrelated strains (Leonardo & Markey, 2008). Until very recently PFGE was regarded as the molecular typing “gold standard” for *S. aureus* (Murchan, Kaufmann, Deplano, de Ryck, Struelens, Zinn, Fussing, Salmenlinna, Vuopio-Varkila, El Solh, Cuny, Witte, Tassios, Legakis, van Leeuwen, van Belkum, Vindel, Laconcha, Garaizar, Haeggman, Olsson-Liljequist, Ransjo, Coombes & Cookson, 2003). Disadvantages relate principally with difficulties in inter-laboratory comparison of results and consequently reliable comparison of strains between regions and internationally (Leonard & Markey, 2008), its time consuming and the expensive cost of the method (Montesinos, Salido, Delgado, Cuervo & Sierra, 2002). In 2003, Murchan et al., in a multicenter study, described a consensus PFGE protocol for typing of strains of MRSA, which resulted in higher intercenter reproductibility, local acceptability and the establishment of a web-based database of harmonized MRSA *Sma*I restriction patterns (Murchan et al., 2003). This protocol has been adapted, with minor modifications, for use in MRSP strains using the same restriction enzyme, *Sma*I (Perreten et al., 2010). MRSA isolates of ST398 cannot be typed with *Sma*I endonuclease due to a modification at the second cytosine of *Sma*I recognition

site (Argudín et al., 2010). Yet, *Cfr*9I and *Xma*I, two *Sma*I-neoschizomers that cut in the same recognition sequence but at different positions can be used instead, allowing studies for outbreak investigations and traceability studies of MRSA ST398 (Argudín et al., 2010).

1.5.4 SCC*mec* typing

As stated before the *mecA* and *mecC* genes are carried on a genetic element, called SCC*mec* (Hiramatsu et al., 2001). This element inserts precisely into the staphylococcal chromosome at *orfX* (Hiramatsu et al., 2001). It can be found in various *Staphylococcus* species yet the original donor remains unknown (Enright, 2003). SCC*mec* elements are highly diverse in their structural organization and genetic content and have been classified into types and subtypes (International Working Group on the Classification of the Staphylococcal Cassette Chromosome Elements [IWG-SCC], 2009). Types are defined by the combination of (i) the type of *ccr* gene complex (Tables 8 and 9) and (ii) the class of the *mec* gene complex (Table 10) and an example is shown in Figure 9 (IWG-SCC, 2009).

Figure 9. Illustration of an SCC*mec* II (adapted from IWG-SCC, 2009).



The *ccr* complex is constituted by the *ccr* gene(s) and surrounding open reading frames (IWG-SCC, 2009). Three distinct *ccr* genes, *ccrA*, *ccrB* and *ccrC*, have been identified (IWG-SCC, 2009). In *S. aureus*, 4 allotypes of *ccrA* and *ccrB* have been found, however in *S. pseudintermedius* and in CoNS others have been described (IWG-SCC, 2009; Zong, Peng & Lü, 2011).

Table 8. Currently classified *ccr* gene complexes (adapted from IWG-SCC, 2009).

ccr gene complexes	*ccr* genes	SCC*mec* types carrying the *ccr* gene complexes
Type 1	A1B1	I, IX
Type 2	A2B2	II, IV
Type 3	A3B3	III
Type 4	A4B4	VI, VIII
Type 5	C1	V, VII
Type 6	A5B3	-
Type 7	A1B6	X
Type 8	A1B3	XI

As methicillin-resistance is prevalent in CoNS, these species may serve as reservoirs of SCC*mec* (Zong et al., 2011). SCC*mec* elements are more diverse in CoNS, with new variants of the *ccr* genes continuing to be frequently identified (Zong et al., 2011).

Table 9. Identified *ccr* genes in staphylococci (adapted from Zong et al., 2011).

ccr genes	Staphylococcal species
A1B4	*S. saprophyticus*
A5B3	*S. hominis, S. cohnii, S. pseudintermedius, S. haemolyticus*
A2B2 & C1	*S. epidermidis, S. haemolyticus*
A1B1 & C1	*S. cohnii, S. hominis*
A4B4 & C1	*S. epidermidis*

The *mec* gene complex is constituted by the *mecA* or *mecC*, its regulatory genes, and associated insertion sequences (IS) (IWG-SCC, 2009). The class A *mec* gene complex is the prototype, containing the *mecA* and the complete *mecR1* and *mecI* regulatory genes (IWG-SCC, 2009). Downstream of *mecA* there is the hypervariable region and IS*431* (IWG-SCC, 2009).

Table 10. Currently identified *mec* gene complexes in staphylococci (adapted from IWG-SCC, 2009).

mec gene complexes	Sequence	SCC*mec* types carrying the *mec* gene complexes
Class A	IS*431-mecA-mecR1-mecI*	II, III, VIII
Class B	IS*431-mecA*-Δ*mecR1*-IS1272	I, IV, VI
Class C1	IS*431-mecA*-Δ*mecR1*-IS*431*	VII, X
Class C2	IS*431-mecA*-Δ*mecR1*-IS*431*	V, IX
Class D	IS*431-mecA*-Δ*mecR1*	-
Class E	*blaZ-mecC-mecCR1-mecCI*	XI

Each SCC*mec* type has been further classified into subtypes based on the polymorphisms or variations in J regions within the same *ccr* gene complex and *mec* gene complex combination (IWG-SCC, 2009).

The first MRSA isolated carried an SCC*mec* type I (Enright, 2003). The predominant SCC*mec* type in CA-MRSA is type IV (Vandenesch et al., 2003). The most prevalent SCC*mec* types in MRCoNS are types III, IV and V (Zong et al., 2011). SCC*mec* type III is the predominant type in the *S. sciuri* group, SCC*mec* type IV in *S. epidermidis*, SCC*mec* type V in *S. haemolyticus* and SCC*mec* type VI in *S. hominis* (Zhang, Agidi & LeJeune, 2009; Faria, Conceição, Miragaia, Bartels, de Lencastre & Westh 2014).

The first MRSP strains described in Europe and North America harboured mainly SCC*mec* III and SCC*mec* V, respectively (Sasaki et al., 2007; Black, Solyman Eberlein, Bemis, Woron & Kania, 2009). After, two other cassettes have been identified in MRSP strains, SCC*mec* II-III and SCC*mec* VII, which belong to class A, allotype 3, and class A, allotype 5, respectively

(Descloux, Rossano & Perreten, 2008). SCC*mec* II-III consists of a combination of *S. aureus* SCC*mec* III and *S. epidermidis* SCC*mec* II (Descloux et al., 2008). SCC*mec* IV has also been identified, recently, in MRSP of lineage ST261 (McCarthy et al., 2014). One SCC*mec* element from MRSP lineage CC45 was firstly identified as non-typeable and was later re-classified as a pseudo-SCC*mec* element (ψSCC*mec*$_{57395}$) due to the lack of *ccr* genes (Perreten et al., 2013).

The *mecC* gene is present as part of a type XI SCC*mec* element inserted at *orfX* carrying the regulators *mecI-mecR1*, as part of a class E *mec* complex that shares structural similarity (*mecI-mecR1-mecC-blaZ*) with a *mec* gene complex, containing the *mecB* gene, found in *Macrococcus caseolyticus* (Tsubakishita, Kuwahara-Arai, Baba & Hiramatsu, 2010). The SCC*mec* XI element also includes the recombinase genes *ccrA1B3* (type 8 *ccr*) and arsenic resistance genes (Shore, Deasy, Slickers, Brennan, O'Connell, Monecke, Ehricht & Coleman, 2011).

1.5.5 Whole-genome sequencing (WGS)

WGS is being considered the ultimate tool for epidemiological typing of bacteria and other pathogens, including staphylococci (Struelens & Brisse, 2013). This technique combines two major advantages compared to previous methods: maximal strain discrimination, and link to clinically and epidemiologically relevant phenotypes (Struelens & Brisse, 2013). Furthermore, WGS provides full genomic characteristics of the infectious isolates, including the set of genes linked to antimicrobial resistance (the resistome) and those linked to virulence of the isolates (the virulome) (Sabat et al., 2013; Struelens & Brisse, 2013). The main disadvantage of this typing is its cost, however they are continuously declining (Sabat et al., 2013).

1.5.6 Epidemiology of CoPS

1.5.6.1 Epidemiology of *S. aureus*

The first MRSA described was isolated in England in 1961 and was only resistant to β-lactam antimicrobials (Jevons, 1961). In the following years after the identification of the first case of MRSA infection, this agent spread globally and it has become highly prevalent in hospitals all over the world (Stefani, Chung, Lindsay, Friedrich, Kearns, Westh & MacKenzie, 2012). In general, the highest prevalence (>50%) of MRSA is present in America and Asia and intermediate prevalence (25-50%) is detected in Australia, China and Africa (Stefani et al., 2012). In Europe, there is an enormous variability in the prevalence (0-65%) of MRSA among countries: in the south and southeast region (e.g. Portugal, Italy, Greece, Malta and Romania) rates are generally higher than in the north (e.g. Netherlands, Sweden, Norway and Denmark) (European Antimicrobial Resistance Surveillance Network [EARS-Net], 2014). For a long time, these MRSA infections were limited to the hospital environment and were denominated hospital/healthcare-acquired/associated MRSA (HA-MRSA), and occurred especially

in patients with risk factors such as prolonged hospitalization, prolonged antimicrobial therapy, surgical interventions, patients with weakened immune system and/or with contact with MRSA positive people (Catry, van Duijkeren, Pomba, Greko, Moreno, Pyörälä, Ruzauskas, Sanders, Threlfall, Ungemach, Törneke, Munoz-Maduro & Torren-Edo, 2010). Only a small number of clones were successful in this environment (Stefani et al., 2012). The main HA-MRSA clones belonged to CC5, CC8, CC22, CC30 and CC45 (Enright et al., 2002; Stefani et al., 2012.). The distribution of these clones varies geographically, with some lineages clustered in specific geographical locations (Stefani et al., 2012). For example, the HA-MRSA dominant clones in the United States of America are the ST5-II (USA100), ST5-IV (USA800) and ST8-IV (USA500), in the United Kingdom and Portugal is the ST22-IV (EMRSA-15), while in Germany the CC5 and ST45-IV clones are the most frequent. In South America, Australia and Asia the ST239-III is the main clone (Stefani et al., 2012). In general, the clones CC5 and CC8 are the most prevalent worldwide, and the CC22 is also very spread (Stefani et al., 2012).

In recent years, there have been MRSA infections in healthy young people in the community, without the typical risk factors associated with nosocomial infections (Stefani et al., 2012). The strains responsible for these types of infection are phenotypically and genotypically distinct of HA-MRSA strains; having received the designation CA-MRSA (Otter & French, 2010). There are several definitions for CA-MRSA, but a globally accepted definition is that the isolates that are CA-MRSA are isolated from an outpatient or from a patient on the first 48 hours after admission to the hospital; as long as such a patient does not have history of previous MRSA colonization or infection, or hospitalization, surgical procedures or dialysis in the last year, and does not have permanent catheters (Otter & French, 2010). CA-MRSA clones are genetically diverse, belonging to a wide variety of lineages, and some belong to HA-MRSA clones (Otter & French, 2010). Similarly to nosocomial clones, CA-MRSA clones are also associated with specific geographic locations: USA300 is extremely widespread in the USA, the Southwest-Pacific clone (ST30-IVc) and the Queensland clone (ST93-IVa) in Australia and New Zealand; the Taiwan clone (ST59-IVa, ST59-V) and USA700 (ST72-IVc) in Asia; ST88-IV in Africa and the European clone (ST80-IVc) in Europe (Rolo, Miragaia, Turlej-Rogacka, Empel, Bouchami, Faria, Tavares, Hryniewicz, Fluit, de Lencastre & the CONCORD Working Group, 2012b). However, it has been observed a spill over of *S. aureus* clones from the hospital into the community, including ST22-IVh, ST105-II and ST5-IVc clones (Espadinha, Faria, Miragaia, Lito, Melo-Cristino & de Lencastre, 2013).

In the early 2000s a new category of MRSA emerged in humans due to exposure to livestock (livestock-associated MRSA [LA-MRSA]) (Armand-Lefevre, Ruimy & Andremont, 2005; Voss, Loeffen, Bakker, Klaassen & Wulf, 2005). These LA-MRSA strains were associated with lineage ST398 and were mainly found in pigs (Armand-Lefevre et al., 2005; Voss et al., 2005). Since then MRSA ST398 have been detected in several species, including horses (Van den

Eede, Martens, Lipinska, Struelens, Deplano, Denis, Haesebrouck, Gasthuys & Hermans, 2009), dogs (Nienhoff, Kadlec, Chaberny, Verspohl, Gerlach, Schwarz, Simon & Nolte, 2009), cats (Weiß, Kadlec, Feßler & Schwarz, 2013), cows (Feβler, Scott, Kadlec, Ehricht, Monecke & Schwarz, 2010) and turkeys (Vossenkuhl, Brandt, Fetsch, Käsbohrer, Kraushaar, Alt & Tenhagen, 2014) around the world (Price, Stegger, Hasman, Aziz, Larsen, Andersen, Pearson, Waters, Foster, Schupp, Gillece, Driebe, Liu, Springer, Zdovc, Battisti, Franco, Żmudzki, Schwarz, Butaye, Jouy, Pomba, Porrero, Ruimy, Smith, Robinson, Weese, Arriola, Yu, Laurent, Keim, Skov & Aarestrup, 2012). MRSA ST398 has already been detected in Portugal, as colonizers and as infecting pathogens causing exsudative epidermitis in pigs (Pomba, Hasman, Cavaco, Fonseca & Aarestrup, 2009; Pomba et al., 2010). Three different SCC*mec* types (IV, V and VII-like) and several subtypes (IVa, IVc, Va, Vb, Vc) have been detected in ST398 (Price et al., 2012; Chlebowicz, Bosch, Sabat, Arends, Grundmanna, van Dijla & Buist, 2013). One of these subtypes, with major structural differences in the J1 region, is specifically associated with ST398 PVL-positive strains (Chlebowicz et al., 2013). MRSA ST398 originated in humans as MSSA and then jumped from humans to livestock, loosing the phage-carried human virulence genes (φSa3 phage carrying genes sak [staphylokinase], *chp* [chemotaxis inhibitory protein] and *scn* [staphylococcal complement inhibitor]) and acquiring tetracycline [*tet*(M) gene] and methicillin resistance (Price et al., 2012). Furthermore, a small cluster of avian CC398 strains acquired φAvβ prophage and two genes that belong to the avian-niche-specific accessory gene pool (Price et al., 2012). A rare subclass of MRSA ST398 strains carry the toxin Panton-Valentine leukocidin (PVL) and are tetracycline susceptible (Welinder-Olsson, Florén-Johansson, Larsson, Oberg, Karlsson & Ahrén, 2008; Chlebowicz, Nganou, Kozytska, Arends, Engelmann, Grundmann, Ohlsen, van Dijl & Buist, 2010; Stegger, Lindsay, Sørum, Gould & Skov, 2010), in contrast to the vast majority of known livestock-associated ST398 isolates that are PVL-negative and tetracycline resistant (Wulf, Tiemersma, Kluytmans, Bogaers, Leenders, Jansen, Berkhout, Ruijters, Haverkate, Isken & Voss, 2008).

Another MRSA clone that has jumped from humans to animals is CC5 (Lowder, Guinane, Ben Zakour, Weinert, Conway-Morris, Cartwright, Simpson, Rambaut, Nübel & Fitzgerald, 2009). The clone CC5 is one of the most successful human-associated lineages of *S. aureus*, characterized by its global distribution and frequent emergence of methicillin-resistant strains (Nübel, Roumagnac, Feldkamp, Song, Ko, Huang, Coombs, Ip, Westh, Skov, Struelens, Goering, Strommenger, Weller, Witte & Achtman, 2008). The majority of *S. aureus* isolates from broiler chickens descend of a single human-to-poultry host jump that occurred approximately 38 years ago by a subtype of the human CC5 clonal lineage unique to Poland (Lowder et al., 2009). In contrast to human subtypes of CC5 that cluster geographically, the poultry CC5 clade is distributed in different continents (Lowder et al., 2009). Like in ST398, the poultry CC5 clade has undergone genetic diversification from its human progenitor strain

by acquisition of novel mobile genetic elements (φAvβ prophage) from an avian-specific accessory gene pool, and by the inactivation of several proteins important for human disease pathogenesis (φSa3 phage) (Lowder et al., 2009). Interestingly, an increase in the prevalence of skeletal infections of poultry, a major cause of lameness in the industry, is correlated with the emergence and wide dissemination of the CC5 poultry subtype (Lowder et al., 2009). Of note, CC5 is associated with increased frequency of haematogenous infections in humans, including osteomyelitis (Fowler, Nelson, McIntyre, Kreiswirth, Monk, Archer, Federspiel, Naidich, Remortel, Rude, Brown, Reller, Corey & Gill, 2007), consistent with the largely skeletal tropism of infections caused by the poultry CC5 clade (Lowder et al., 2009). In the United States of America MRSA from cats and dogs were mainly CC5 (USA100), and had genotypic and virulence profiles more similar to each other than to those of horses (Lin, Barker, Kislow, Kaldhone, Stemper, Pantrangi, Moore, Hall, Fritsche, Novicki, Foley & Shukla, 2011). This clone has also been detected in horses in the United States of America (Lin et al., 2011) and from bovine milk in Japan (Hata, Katsuda, Kobayashi, Uchida, Tanaka & Eguchi, 2010). In Japan, this lineage, uncommon among bovine isolates but common in human MRSA isolates, could have been introduced by humans due to the entrance of unauthorized personnel and ambulatory patients in dairy farms (Hata et al., 2010).

Contrary to CC5 and ST398, MRSA lineage CC97, a major bovine *S. aureus* complex, jumped from livestock-to-human (Spoor, McAdam, Weinert, Rambaut, Hasman, Aarestrup, Kearns, Larsen, Skov & Fitzgerald, 2013). Methicillin resistance was acquired by human CC97 clones subsequent to the host jump from cows (Spoor et al., 2013). Furthermore, human CC97 clades also acquired the φSa3 phage and some strains had the arginine catabolite mobile element (ACME), characteristic of CA-MRSA clones that contributes to enhanced survival during infection (Spoor et al., 2013).

In 2011 a novel variant of *mecA* was identified in *S. aureus* from cattle (Garcia-Alvarez et al., 2011). This variant was identified in MRSA strains of the CC130 (Shore et al., 2011). After the first description it has been described in humans, and a range of other animal species in Denmark, France, The Netherlands, Ireland, Germany, Belgium, and the United Kingdom (Garcia-Alvarez et al., 2011; Becker, Larsen, Skov, Paterson, Holmes, Sabat, Friedrich, Köck, Peters & Kriegeskorte, 2013; Loncaric, Kübber-Heiss, Posautz, Stalder, Hoffmann, Rosengarten & Walzer, 2013). This subtype was originally designated $mecA_{LGA251}$ but it has been renamed *mecC* and shares 70% nucleotide identity with the *mecA* gene (Becker et al., 2013). The frequency of human infection with LA-MRSA CC130 is even lower than for LA-MRSA ST398 (Guardabassi, Larsen, Weese, Butaye, Battisti, Kluytmans, Lloyd & Skov, 2013). However, there are potential diagnostic problems associated with the detection of the *mecC* gene and the emergence of novel MRSA lineages in animals (Guardabassi et al., 2013). Isolates carrying the *mecC* gene are not detected by conventional confirmatory tests

(i.e. standard *mecA* PCR and PBP2a latex agglutination test) thereby creating the potential for misdiagnosis and inappropriate therapy (Guardabassi et al., 2013).

Several epidemiological studies have described the incidence and characteristics of MRSA in companion animals (Weese & van Duijkeren, 2010). Usually MRSA clones found in dogs and cats tend to be those that predominate in humans in a given region (Weese & van Duijkeren, 2010). For example, Moodley and colleagues (2006) found that most MRSA strains found in cats and dogs in the United Kingdom and Ireland belonged to the ST22-IV (EMRSA-15) clone, which is, as mentioned above, one of the predominant clones in human hospitals in these two countries (Moodley, Stegger, Bagcigil, Baptiste, Loeffler, Lloyd, Williams, Leonard, Abbott, Skov, & Guardabassi, 2006). In Portugal, dogs and cats have also been showed to carry MRSA ST22-IV, which circulates in the human population in this country (Coelho, Torres, Radhouani, Pinto, Lozano, Gómez-Sanz, Zaragaza, Igrejas & Poeta, 2011; Couto, Pomba, Moodley & Guardabassi, 2011). MRSA ST239-III has been found in small animals in Australia (Malik, Coombs, O'Brien, Peng & Barton, 2006), the most frequent clone in human hospitals (Stefani et al., 2012). This strongly suggests that the principal origin for MRSA colonization and infection in companion animals is through contact with humans infected with or carrying MRSA (Leonard & Markey, 2008). Dogs and cats can then serve as reservoirs and act as a source of re-infection or re-colonization (Leonard & Markey, 2008). The distribution of MRSA clones in horses is a little bit different from dogs and cats (Weese & van Duijkeren, 2010). The first MRSA strains isolated from horses belonged to ST8 and other STs within the CC8 (Weese & van Duijkeren, 2010). In the United States of America, CC8 was a typical equine clone (USA500), which emerged as an important HA-MRSA clone in this country despite being responsible for a small percentage of infections in humans (Weese, 2010; Lin, Barker, Kislow, Kaldhone, Stemper, Pantrangi, Moore, Hall, Fritsche, Novicki, Foley & Shukla, 2011). The predominance of this human epidemic clone in horses suggested that it adapted to this animal species (van Duijkeren & Weese 2010). However in Europe, ST398 was the predominant clone among horses suggesting they acquired this clone directly or indirectly from food animals (van Duijkeren & Weese 2010).

1.5.6.2 Epidemiology of *S. pseudintermedius*

The first phenotypic MRSP strains were isolated in France in the mid-1980s from healthy dogs and dogs with pyoderma (Pellerin, Bourdeau, Sebbag & Person, 1998). However, only in 1999 was the first *mecA*-positive strain detected in United States of America from a dog with pyoderma (Gortel, Campbell, Kakoma, Whittem, Schaeffer & Weisiger, 1999). In Europe, only in 2005 were identified the first *mecA*-positive *S. pseudintermedius* strains (Loeffler, Linek, Moodley, Guardabassi, Sung, Winkler, Weiss & Lloyd, 2007). MRSP strains are particularly resistant to many different classes of antimicrobials, such as β-lactams, aminoglycosides, fluoroquinolones, tetracyclines, lincosamides, macrolides, folate pathway inhibi-

tors, and phenicols, thus limiting the therapeutic options (Frank & Loeffler, 2012). There are two major MRSP clones identified in Europe and North America and they are designated ST71-II-III (Europe) and ST68-V (North America), respectively (Black, Eberlein, Solyman, Wilkes, Hartmann, Rohrbach, Bemis & Kania, 2011). ST71 isolates have both the *blaI/blaR1* and *mecI/mecR1* regulatory mechanisms controlling the expression of the *mecA* gene, leading to low levels of PBP2a (Black et al., 2011). ST68 isolates, however, only have the *blaI* and *blaR1* regulating *mecA* expression, which results in high constitutive *mecA* expression even in the presence of only low concentrations of oxacillin (Black et al., 2011). Other clonal lineages have been reported to be predominant in some countries (Perreten, Chanchaithong, Prapasarakul, Rossano, Blum, Elad & Schwendener, 2013). In South China ST4, ST5, and ST95 were the dominant sequence types found in a study in 2012 (Feng, Tian, Lin, Luo, Zhou, Yang, Deng, Liu & Liu, 2012). In North China, on the other hand, the most prevalent genotypes detected were ST71-t06-II-III, followed by ST5-t19, ST126-III and ST6-t02-V (Wang, Yang, Logue, Liu, Cao, Zhang, Shen & Wu, 2012). In South America only recently MRSP strains were described, belonging to the European clone (ST71-III) (Quitoco, Ramundo, Silva-Carvalho, Souza, Beltrame, de Oliveira, Araújo, Del Peloso, Coelho & Figueiredo, 2013). In Israel and Thailand, dogs and cats carried or were infected with specific MRSP strains that were non-typeable by *Sma*I PFGE and SCC*mec* typing using the Kondo method and were found to belong to a specific lineage, CC45 (ST45, ST57, ST85 and ST179), and carried a novel pseudo-SCC*mec* element with no *ccr* genes (Perreten et al., 2013).

1.5.7 Epidemiology of CoNS

An international study performed in Europe, Asia and Latin America revealed that approximately 70% of *S. epidermidis* strains circulating in the hospital environment are resistant to methicillin and a high percentage are also resistant to other antimicrobial classes (Sanches, Mato, de Lencastre, Tomasz, Nunes, Alves, Miragaia, Carriço, Couto, Bonfim, Aires de Sousa, Oliveira, Gomes, Vaz, Fernandes, Verde, Ávila, Antunes, Sá-Leão, Almeida, Melter, Chung, Brandileone, Castañeda, Cocuzza, Echaniz-Aviles, Heitmann, Hortal, Hryniewicz, Jia, Kikuchi, Konkoly-Thege, Kristinsson, Liñares, Rossi, Savov, Schindler, Solorzano-Santos, Totsuka, Venditti, Villari, Westh, Wu & Zanella, 2000). ST2 was identified in 13 different countries across 4 continents (Miragaia, Thomas, Couto, Enright & de Lencastre, 2007). ST35 and ST57 were two lineages only found in Portugal (Miragaia et al., 2007). The most frequently found STs among carriage isolates was ST2, followed by ST59, ST22 and ST5; while among infection strains was ST2 followed by ST23 and ST59 (Miragaia et al., 2007). There was more genetic diversity in methicillin-susceptible *S. epidermidis* than in methicillin-resistant *S. epidermidis* (MRSE), however CC2 was the predominant clonal lineage in both (Miragaia et al., 2007). This genetic diversity may be due to the need for adaptation to different environments in hospital and community settings, which in turn leads to in-

creased frequency of horizontal gene transfer and dissemination of mobile genetic elements (Miragaia et al., 2007). Higher recombination rates were observed within CC2 due to a result of exclusive restriction modification systems between different *S. epidermidis* lineages (Miragaia et al., 2007). This mechanism, would actually favour the recombination between *S. epidermidis* strains belonging to CC2 opposed to recombination between strains of CC22 and strains of other lineages (Miragaia et al., 2007). Hospital *S. epidermidis* has an epidemic population that evolves rapidly by means of recombination and frequent transfer of genetic mobile elements, including SCC*mec* (Miragaia et al., 2007). In fact a much higher genetic diversity in SCC*mec* was observed among hospital isolates (Rolo, de Lencastre & Miragaia, 2012). In the hospital a large reservoir of SCC*mec* types exist in other CoNS, and this may contribute to the genetic diversity observed in *S. epidermidis* (Rolo et al., 2012). Specific physiological conditions during infection and stress imposed by the hospital environment can promote SCC*mec* excision/acquisition and dissemination in the *S. epidermidis* hospital population (Rolo et al., 2012). SCC*mec* transfer may also be promoted during biofilm formation (Rolo et al., 2012). Hospital isolates, particularly those belonging to CC2, were associated with the presence of the *ica* operon and the ACME (Rolo et al., 2012). Very recently CC2 has been renamed CC5 (Rolo et al., 2012). CC5 is now the predominant clone in the hospital and community settings (Rolo et al., 2012). The great majority of these isolates carry SCC*mec* type IV, which probably confers advantages and has no fitness cost in either environment (Rolo et al., 2012).

S. epidermidis has been isolated from small animals in Australia (Malik et al., 2006). Two STs were found, ST43 and ST60, which carried SCC*mec* IVb (Malik et al., 2006). Up until 2006, those STs were unique in the MLST database and the authors suggested that those *S. epidermidis* strains were specific to cats and dogs (Malik et al., 2006). In Portugal, *S. epidermidis* has been described in birds of prey, belonging to ST35 (CC2) (Sousa et al., 2014). A recent study examining *S. aureus* and CoNS from pig farms identified common SCC*mec* types shared in *S. aureus* and *S. epidermidis* from the same environmental niche, indicating the possibility of interspecies exchange of SCC*mec* (Tulinski, Fluit, Wagenaar, Mevius, van de Vijver & Duim, 2012).

1.6 The problem of *S. pseudintermedius*

One of the most common diseases caused by *S. pseudintermedius* is pyoderma in dogs (Figure 10) (Nuttall et al., 2013). Pyoderma is usually secondary to atopic dermatitis, a pruritic allergic skin disease that results in disrupted skin barrier and predisposition for secondary *S. pseudintermedius* infections (Nuttall et al., 2013). Pyoderma is usually treated with antimicrobials (Frank et al., 2012).

Figure 10. A dog with *S. pseudintermedius* pyoderma secondary to atopic dermatitis (original photo).

The selective pressure imposed by the long-term administration of antimicrobials can lead to the development of bacterial resistance that could potentially be transmitted to human pathogens, like *S. aureus*. The recent emergence of MRSP has complicated considerably the treatment of infections caused by these bacteria. MRSP have become virtually resistant to all the antimicrobials approved for administration in companion animals, which has led to ethical concerns about the use of antimicrobials classified by the World Health Organization as "critically important" for human medicine (Frank et al., 2012). There has always been an interest in developing alternative options to antimicrobials, however since the appearance of multidrug-resistant *S. pseudintermedius*, this interest has been renewed. Several approaches can be chosen, like phage therapy, antivirulence drugs or vaccines.

1.6.1 Phage therapy

Bacteriophages or phages are viruses that invade bacterial cells and may disrupt bacterial metabolism and eventually cause lysis (Sulakvelidze, Alavidze & Morris, 2001). Recently, with the significant increase in the number of deaths caused by multidrug-resistant bacteria, including staphylococci, there was a renewed interest in phage therapy both in human and veterinary medicine (Thiel, 2004; Mann, 2008; Kaźmierczak, Górski & Dąbrowska, 2014).

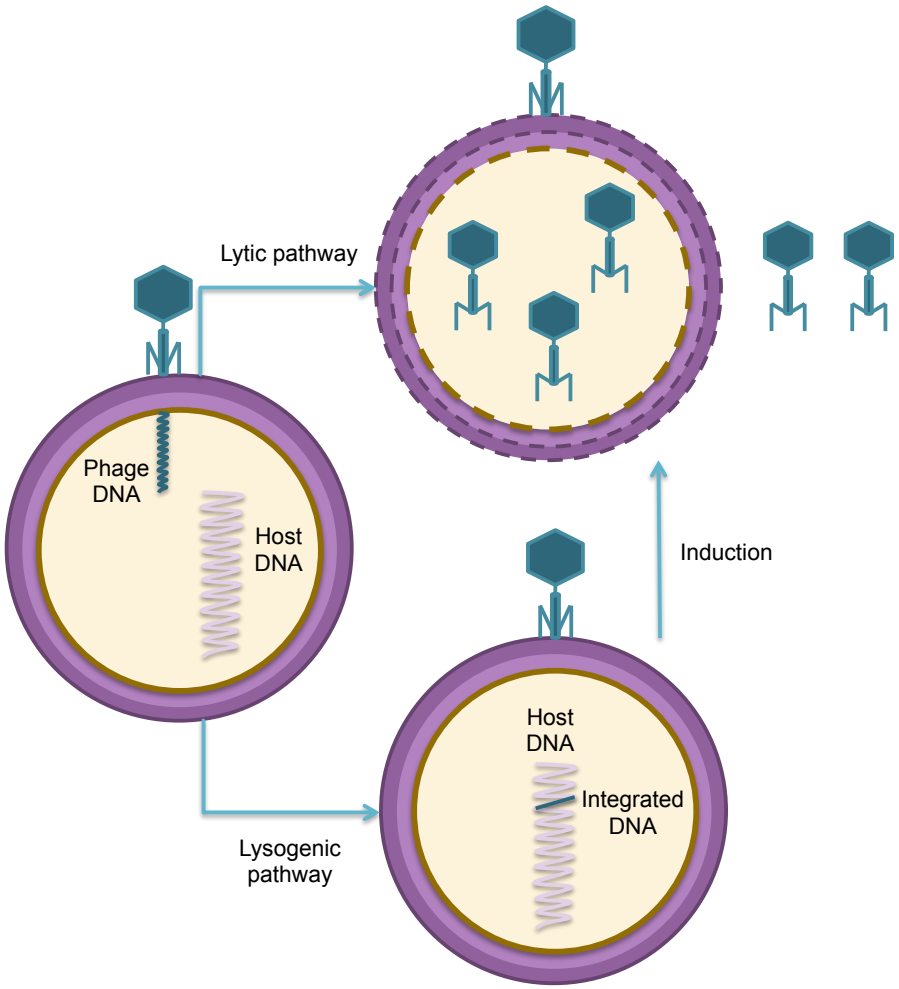
Phages against staphylococci belong to the *Caudovirales* order: phages with an icosahedral head, tube-like tail and linear, double-stranded DNA (Figure 11) (Ackermann, 2007). The members of this order are divided (Kropisinski, 2006):

a) Morphologically, based on the length and complexity of their tales;

b) Functionally, based on their effect upon host infection (lytic or lysogenic).

Functionally, phages can be lytic or lysogenic/temperate (Kropisinski, 2006). Other authors divide further and consider phages into obligately lytic, temperate and chronic (Mann, 2008). Obligately lytic phages lead exclusively to cell death and release of progeny phages, since they can only undergo the lytic pathway (Kropisinski, 2006; Mann, 2008).

Figure 11. Functional characterization of the *Caudovirales* order (adapted from Kropisinski, 2006).



The lysogenic or temperate phages may also lead to cell death, through the lytic pathway, or if the phage lytic functions are repressed, the virus genome coexists in a stable form (i.e. as a prophage) within its host (Kropisinski, 2006; Mann, 2008). In these cases, the virus DNA can either be integrated in the host DNA or remain separate (Kropisinski, 2006). A chronically infecting phage can release progeny into the extracellular environment without killing its host that can continue to grow and divide (Mann, 2008). Conventionally, lytic phages offer the greatest therapeutic potential since they invariably cause death of the bacterial host (Mann, 2008; Kaźmierczak et al. 2014). Temperate phages, on the other hand, are not suitable for phage therapy because they may not kill the bacterial host (Mann, 2008). Furthermore, temperate phages may actually carry genes that turn the bacteria more virulent, in a process called lysogenic conversion (Kropisinski, 2006). Temperate phages encode a large proportion of *S. aureus* virulence factors (e.g. the immune evasion cluster [IEC]) and provide the pathogen with a large variety of toxins, allowing, for example, escaping the host immune system (Deghorain & Melderen, 2012).

One of the first phage preparations against *S. aureus* was produced by D'Herelle's commercial laboratory in Paris and was called Bacté-staphy-phage (Sulakvelidze et al. 2001). In the 1940s, the Eli Lilly Company (Indianapolis, Ind.) was producing therapeutic phages in the United States of America (Sulakvelidze et al. 2001). These preparations consisted of phage-

lysed, bacteriologically sterile broth cultures of the targeted bacteria (e.g. Staphylo-lysate) or the same preparations in a water-soluble jelly base (e.g. Staphylo-jel) (Sulakvelidze et al. 2001). However, as stated before, these productions were discontinued and only institutions localized in Eastern Europe were actively involved in therapeutic phage research and production (Sulakvelidze et al. 2001; Kaźmierczak et al. 2014). Indeed, in Georgia, phage products are available in pharmacies with a prescription (Kaźmierczak et al. 2014).

Studies using phage therapy against staphylococcal infections seem promising. In the 1970s, Sakandelidze and colleagues administered phages against *Staphylococcus*, *Streptococcus*, and *Proteus*, subcutaneously or via a surgical drain daily for 5–10 days to human patients, leading to an improvement in 92% of the investigated cases (Sakandelidze & Meipariani, 1974). In the 1980s, Ślopek and colleagues observed improvement in 75% of infected ulcerated varicose vein cases and in 100% of cases of gastrointestinal infections, pericarditis, and furunculosis, caused by *Staphylococcus* species (Ślopek, Durlakowa, Weber-Dabrowska, Kucharewicz-Krukowska, Dabrowski & Bisikiewicz, 1983; Ślopek, Weber-Dabrowska, Dabrowski & Kucharewicz-Krukowska, 1987). Overall, in studies reported by the Hirszfeld Institute of Immunology and Experimental Therapy, staphylococcal phages administered by different routes, topically, orally, or both, were effective in the treatment of bacterial infections (Sulakvelidze et al. 2001).

Phage therapy has also been applied to animals. In fact, two companies offer phage lysates against *Staphylococcus* or phage cocktails applicable in veterinary medicine. One of them is a *S. aureus* phage lysate, which is actually a mixture of a *S. aureus* bacterin and a phage lysate, SPL® (Delmont Laboratories Inc., Swarthmore, United States of America), which was used very frequently in the 80s and was considered highly effective for the treatment of idiopathic pyoderma caused by *S. (pseud)intermedius* in dogs (DeBoer, Moriello, Thomas & Schultz, 1990). The other is an antistaphylococcal phage lysate (Stafal®, Sevapharma, Praha, Czech Republic) recommended for topical applications in veterinary medicine, available in Czech Republic (Kaźmierczak et al. 2014). However, poor results were obtained in a study of the efficacy and pharmacokinetics of phage therapy to treat subclinical *S. aureus* mastitis in dairy cattle in a placebo-controlled multisite trial (Gill, Pacan, Carson, Leslie, Griffiths & Sabour, 2006a), due to an apparent inactivation of phage within the mammary gland.

Phage therapy seems very promising, however, some scientific and logistical challenges remain (Thiel, 2004). Wild-type phage particles are rapidly eliminated by the body's reticuloendothelial (mononuclear phagocyte) system (Thiel, 2004). So in order to enhance the efficacy of treatment, long-circulating mutants (Merril, Biswas, Carlton, Jensen, Creed, Zullo & Adhya, 1996) must be chosen, or wild-type virions must be protected with a non-immunogenic polymer such as polyethylene glycol (Kim, Cha, Jang, Klumpp, Hagens, Hardt, Lee & Loessner, 2008). Some studies observed inhibitory effect of whey proteins on phage-host interactions (Gill, Sabour, Leslie & Griffiths, 2006). The development of phage-

neutralizing antibodies is another problem that has been documented after parenteral administration, which may obstruct phage effectiveness in lysing targeted bacteria (Kucharewicz-Krukowska & Slopek, 1987). Another concern regarding the therapeutic use of lytic phages is development of phage resistance (Sulakvelidze et al. 2001). Bacterial resistance to phages will undeniably develop, although some authors state that the rate of developing resistance to phages is approximately 10-fold lower than that to antimicrobials (Carlton, 1999). This resistance against phages can be partially avoided by using numerous phages in one preparation and/or in conjunction with antimicrobials (Sulakvelidze et al. 2001). In fact, phage therapy and antimicrobial therapy, given together, are synergistic (Kutateladze & Adamia, 2010). Moreover, like bacteria but unlike antimicrobials, phages mutate and so can develop to counter phage-resistant bacteria (Matsuzaki, Rashel, Uchiyama, Sakurai, Ujihara, Kuroda, Ikeuchi, Tani, Fujieda, Wakiguchi & Imai, 2005).

1.6.2 Antivirulence therapy

To infect animals, bacteria need to express certain factors, virulence determinants that allow them to enter and damage the host (Defoirdt, 2013). As virulence determinants are essential for infection, preventing bacteria from producing these factors will be a good way of controlling bacterial disease (Defoirdt, 2013). Antivirulence therapy is, therefore, based on the ability of disarming pathogens rather than killing them (Defoirdt, 2013). A thorough understanding of the bacterial pathogenesis is consequently needed to develop antivirulence drugs. There are two main ways of interfering with virulence (Defoirdt, 2013):

a) Inhibiting specific virulence factors, such as secretion systems or toxins;

b) Interfering with regulatory mechanisms that control the expression of virulence factors, such as quorum sensing and host-pathogen signalling.

Both strategies have been used against staphylococci and some examples are present in Table 11 (Ragle, Karginov & Wardenburg, 2010; Qiu, Luo, Wang, Dong, Li, Leng, Zhang, Dai, Zhang, Niu & Deng, 2011a; Qiu, Luo, Dong, Wang, Li, Wang, Deng, Feng & Deng, 2011b; Wang, Qiu, Dong, Li, Luo, Dai, Zhang, Leng, Niu, Zhao & Deng, 2011; Qiu, Niu, Wang, Xing, Leng, Dong, Li, Luo, Zhang, Dai, Luo & Deng, 2012; Dong, Qiu, Wang, Li, Dai, Zhang, Wang, Tan, Niu, Deng & Zhao, 2013).

Table 11. Examples of antivirulence drugs tested against staphylococcal specific virulence factors.

Compound	Virulence target	Model of infection	Reference
Apigenin (Extracted from parsley)	Alpha-hemolysin	*In vitro* A549 cells, *in vivo* mouse model of pneumonia	Dong et al. 2013
β-Cyclodextrin derivate IB201	Alpha-hemolysin	*In vivo* murine model of pneumonia	Ragle et al. 2010
Chrysin	Alpha-hemolysin	*In vitro* A549 cells and *in*	Wang et al.

(Present in honey, propolis and many plant extracts)		*vivo* mouse model of pneumonia	2011
Isoalantolactone (Present in *Inula helenium* – Compositae)	Alpha-toxin	*In vivo* mouse model of pneumonia	Qiu et al. 2011a
Capsaicin (Present in red chilli - *Capsicum annuum*)	Alpha-toxin	*In vitro* A549 cells, *in vivo* mouse model of pneumonia	Qiu et al. 2012
Menthol (Present in plants of the *Mentha* species)	Alpha-hemolysin, enterotoxins A and B, toxic-shock syndrome toxin 1	*In vitro*	Qiu et al. 2011b

Note – A549 cells are human alveolar epithelial cells.

Several compounds tested against *S. aureus* have shown promising *in vitro* and *in vivo* results. Interestingly, most of these compounds were developed against one toxin, designated alpha-toxin or alpha-hemolysin. Most of them inhibited the expression of alpha-toxin/alpha-hemolysin and prevented alveolar cell injury (Qui et al. 2011a; Qui et al. 2011b; Qui et al. 2012). Menthol was the only compound that could inhibit the expression of several virulence genes, α-hemolysin, enterotoxins A and B, and toxic shock syndrome toxin 1 in *S. aureus* (Qiu et al. 2011b).

There are also several studies reporting antivirulence drugs testes against virulence regulatory mechanisms (Table 12) (Khodaverdian, Pesho, Truitt, Bollinger, Patel, Nithianantham, Yu, Delaney, Jankowsky & Shoham, 2013; Long, Mead, Hendricks, Hardy & Voyich, 2013; Nielsen, Månsson, Bojer, Gram, Larsen, Novick, Frees, Frøkiær & Ingmer, 2014).

Table 12. Antivirulence drugs tested against staphylococci virulence regulatory mechanisms.

Compound	Virulence regulatory mechanism	Model of infection	Reference
Solonamide B (Isolated from the marine bacterium *Photobacterium halotolerans*)	*agr*	*In vitro* rabbit erythrocytes and human neutrophils	Nielsen et al. 2014
18β-Glycyrrhetinic acid (isolated from the licorice root *Glycyrrhiza* spp.)	*saeR*	*In vivo* mouse model of skin and soft tissue infection	Long et al. 2013
Diflunisal	*agr*	*In vitro* rabbit blood	Khodaverdian et al. 2013

Solonamide B was the first compound produced naturally by a Gram-negative marine bacterium, that interfered with *agr* and affected both RNAIII and AgrA controlled virulence gene

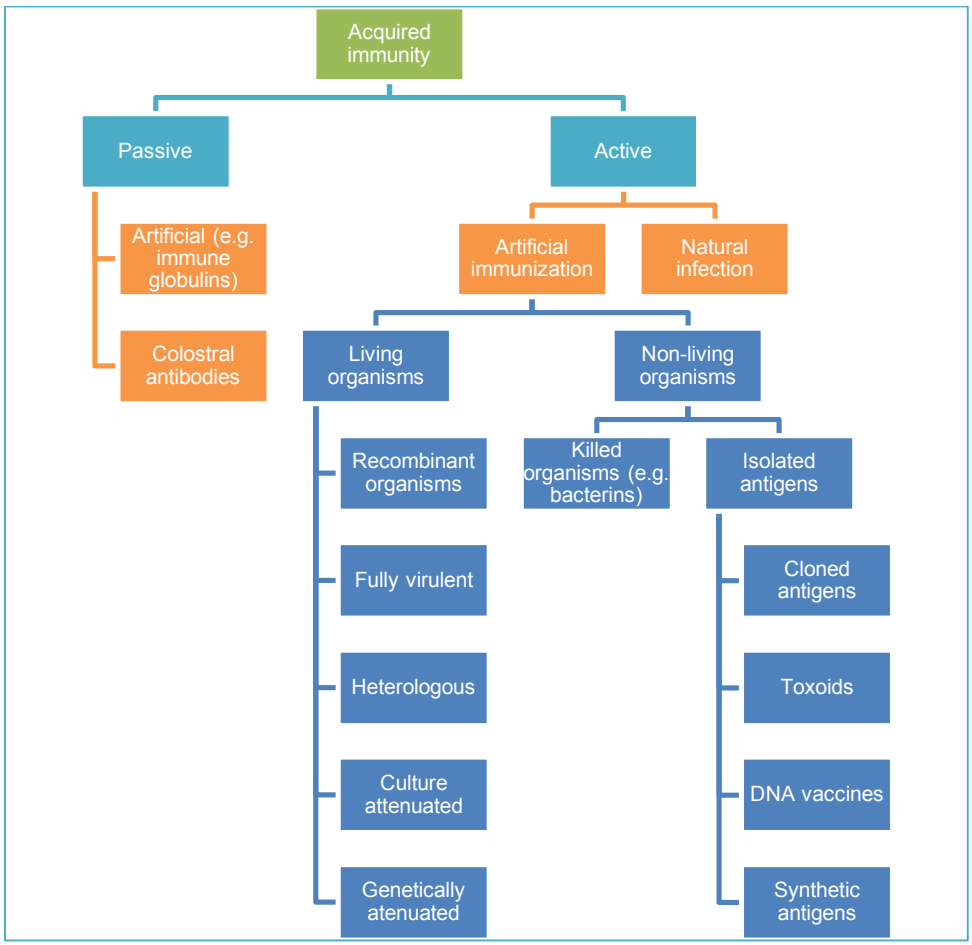
expression in *S. aureus* (Nielsen et al. 2014). One the other hand, 18β-Glycyrrhetinic acid was isolated from a root (*Glycyrrhiza* spp.) and resulted in decreased expression of *saeR* and *hla* and significantly reduced skin lesion size in murine models (Long et al. 2013). Diflunisal is actually a nonsteroidal anti-inflammatory drug but also inhibits the expression of the regulatory mechanism *agr* by binding to AgrA protein (Khodaverdian et al. 2013).

A main advantage of antivirulence therapy, comparing to antimicrobial treatment, is that there will be less interference with non-target organisms (i.e. the commensal microbiota), as it specifically targets virulence factors or virulence gene regulation (Khodaverdian et al. 2013). One other advantage is that antivirulence therapy will only pose selective pressure under conditions in which the virulence genes are required, therefore the tendency to develop resistance and spread will probably also be lower, but not absent (Defoirdt, Boon & Bossier, 2010). However, some resistance mechanisms that bacteria have acquired during exposure to antimicrobials could potentially give resistant to antivirulence agents (Defoirdt, 2013).

1.6.3 Vaccines

Vaccination is regarded as the most efficient and cost-effective method for the control of infectious diseases in humans and animals and they can become immune by two methods: passive and active immunization (Figure 12) (Tizard, 2009).

Figure 12. Classification of the different types of active and passive immunization (adapted from Tizard, 2009).



Passive immunization is the transfer of immunity from one resistant animal to a susceptible one (Tizard, 2009). This type of immunization, also called therapeutic vaccination, gives immediate protection against infection, however this protection declines and the animal ultimately becomes susceptible again (Tizard, 2009). Active immunization on the other side involves administration of an antigen to an animal, so that it builds an immune response (Tizard, 2009). The disadvantage with this immunization, also called prophylactic vaccination, is protection is not conferred immediately (Tizard, 2009). However, once an immune response is established it can be long lasting and capable of re-stimulation (Tizard, 2009).

1.6.3.1 Passive Immunization

This type of immunization requires that antibodies are produced in a donor animal by active immunization and then the antibodies are transferred to the recipient to confer immediate protection (Tizard, 2009). Serum containing these antibodies may be produced against a variety of pathogens, including *Staphylococcus* species (Spellber & Daum, 2012). Several passive immunization vaccines against *S. aureus* have been tested in human clinical trails (Table 13) (Jansen, Girgenti, Scully & Anderson, 2013).

Table 13. Overview of passive immunization vaccines against *S. aureus* (adapted from Otto, 2011; Proctor, 2012; Jansen et al. 2013).

Name	Company	Target
AltaStaph®	Nabi	Capsular polysaccharides types 5 and 8 (CP5, CP8)
Aurexis/Tefibazumab®	Inhibitex	Clumping factor A (ClfA)
Aurograb®	NeuTec/Novartis	ABC Transporter
Pagimaximab®	Biosynexus	Lipoteichoic acid
ETI-211	Elusys/Pfizer	Protein A (Spa)
KBSA301	Kenta Biotech	Alpha toxin
MEDI4893	Medimmune LLC	Alpha toxin
Staphyban®	Berne	Whole cell vaccine and alpha toxin toxoid
Veronate®	Inhibitex	Clumping factor A (ClfA) from *S. aureus* and Serine-aspartate repeat-containing protein G (SdrG) from *S. epidermidis*

These vaccines were used in an attempt to help treating already established *S. aureus* infections, however efficacy in humans was not seen with any of the vaccines tested (Otto, 2010; Proctor, 2012; Jansen et al., 2013).

The intent of passive immunotherapy is to inject enough preformed antibodies to bind and neutralize antigens (commonly toxins) and protect the host (Otto, 2010). So investigators aim

at eliminating major *S. aureus* virulence factors, such as toxins, instead of the whole bacteria (Otto, 2010). However, given the plasticity of staphylococcal toxins and virulence determinants, a passive immunization vaccine should combine antibodies against several different virulence factors (Otto, 2010). As such, investigators should focus on the study of staphylococcal pathogenesis to determine which virulence factors are essential for infection and therefore should be the target of passive immunotherapy (Otto, 2010).

1.6.3.2 Active Immunization

In active immunization, the immune system slowly develops its response against the antigen but this response is usually long lasting (Tizard, 2009). An idyllic vaccine for active immunization should induce a prolonged strong immunity, without adverse side effects (Tizard, 2009). Other characteristics must also be present (Tizard, 2009):

a) The antigen must be provided efficiently so that antigen-presenting cells can process it and release the proper cytokines;

b) Both T and B cells should be stimulated so that memory cells are created;

c) The memory and effector T cells should recognize several epitopes, to avoid individual variations in major histocompatibility complex (MHC) class II polymorphism;

d) The antigens should stimulate protection, through memory T cells, for as long as possible.

Several human active immunization vaccines have been developed against staphylococci (Table 14) (Otto, 2011; Proctor, 2012; Jansen et al. 2013).

Table 14. Overview of active immunization vaccines against *S. aureus* in humans (adapted from Otto, 2011; Proctor, 2012; Jansen et al. 2013).

Name	Company	Target
Nabi Penta Staph®	GlaxoSimthKline	Capsular polysaccharides 5 and 8 (CP5, CP8), wall techoic acid, non-toxic mutants of Panton-Valentine leukocidin (PVL) and alpha-hemolysin (Hla)
NDV3®	NovaDigm Therapeutics	Adhesion protein fragment from *Candida albicans* with similar structure to Clumping factor A (ClfA)
SA3Ag	Pfizer	Capsular polysaccharides 5 and 8 (CP5, CP8), recombinant monoclonal antibodies against Clumping factor A (rmClfA)
SA4Ag	Pfizer	Capsular polysaccharides 5 and 8 (CP5, CP8), recombinant monoclonal antibodies against Clumping factor A (rmClfA) and Manganese-transporter C (MntC)
SA75	Vaccine Research International PLC	Whole cell vaccine
SAR279356	Sanofi Pasteur	Polymeric N-acetylglucosamine (PNAG)

StaphVAX®	Nabi	Capsular polysaccharides 5 and 8 (CP5, CP8), conjugated to *Pseudomonas aeruginosa* exotoxoid
STEBVax®	Integrated BioTherapeutics/NIAID	Enterotoxin B (SEB)
Unnamed	Novartis	Iron-regulated surface determinant A (IsdA), Iron-regulated surface determinant B (IsdB), Serine-aspartate repeat-containing protein D (SdrD), Serine-aspartate repeat-containing protein E (SdrE)
Unnamed	Novartis	Ferrichrome-binding protein (FhuD2), Ess extracellular A and B (EsxAB), alpha-hemolysin (Hla), Sur-2
V710®	Merck	Iron-regulated surface determinant B (IsdB)

All of these vaccines were efficient in protecting animal models against *S. aureus* infections, however, most failed to give significant protection in humans (Otto, 2010). There are several interpretations to why these vaccines failed (Proctor, 2012; Jansen et al., 2013):

a) Antibodies against one single antigen may not be sufficient to protect from staphylococcal infections;

b) The antibodies developed may be opsonic but may not lead to the destruction of the phagocyted bacteria;

c) Antibody avidity has not been shown;

d) Competing anti-idiotype antibodies (e.g. antibodies raised against capsular polysaccharides and against PNAG) may interfere with the success of the vaccine;

e) Antibodies raised against the capsule may select for hyperadhesive strains, which may enhance biofilm formation and increase virulence;

f) Staphylococcal protein A (Spa) and *Staphylococcus aureus* binder of immunoglobulin G (Sbi) proteins may interfere with antibodies;

g) Some staphylococcal strains do not produce virulence determinants to which the vaccine was developed (e.g. not all *S. aureus* produce capsule);

h) Opsonic antibodies may not give more protection than the protection already existent in non-vaccinated individuals.

In spite of the problems found in human clinical trials, new attempts to develop vaccines based on active immunization are on going and will hopefully be successful (Otto, 2010). These vaccines try to overcome the problems found with the first vaccines developed and are based on research efforts in selecting the best vaccine targets (Otto, 2010).

In animals there are also vaccines that were designed for active immunization (Table 15). Some of them are even commercialized although its efficacy is a matter of debate (Pereira et al., 2011).

Table 15. Overview of active immunization commercially available vaccines against staphylococci in animals.

Name	Company	Target	Species to which the vaccine was developed
Lysigin®	Boehringer Ingelheim Vet-medica	Whole-cell vaccine	Bovine
Mastivac®	Laboratorios Ovejero S. A.	Whole-cell vaccine	Bovine
Startvac®	Laboratorios Hipra S. A.	Whole-cell vaccine	Bovine
Vimco®	Laboratorios Hipra S. A.	Whole-cell vaccine	Caprine
SPL®	Delmont Laboratories Inc.	Whole-cell vaccine and phage lysate	Canine

1.6.3.3 Methods for the identification of novel staphylococcal antigens for vaccine development

First generation vaccines were developed based on the Pasteur's strategy, which was to isolate, inactivate or attenuate, and inject the pathogenic microorganism (Movahedi and Hampson, 2008; Serruto and Rappuoli, 2006; Bagnoli, Baudner, Mishra, Bartolini, Fiaschi, Mariotti, Nardi-Dei, Boucher & Rappuoli, 2011; Prachi, Biagini & Bagnoli, 2012). During most of the 20th century this was the approach used for the development of novel bacterial vaccines and today there are still several vaccines that are based on killed or live-attenuated microorganisms (Movahedi and Hampson, 2008; Serruto and Rappuoli, 2006; Bagnoli et al., 2011; Prachi et al., 2012). A second-generation of vaccines has been created based on the use of recombinant proteins and rationally attenuated strains (Movahedi and Hampson, 2008; Bagnoli et al., 2011; Prachi et al., 2012). However, novel approaches in the last decades enabled the identification of third-generation vaccine candidates (Movahedi and Hampson, 2008; Bagnoli et al., 2011; Prachi et al., 2012). This approach has been termed "Reverse Vaccinology" and is based in recent technologies like genomics, transcriptomics or proteomics (Movahedi and Hampson, 2008; Serruto and Rappuoli, 2006; Bagnoli et al., 2011; Prachi et al., 2012). There are limitations to these approaches and these will be discussed within each strategy.

1.6.3.3.1 First-generation approach or Pasteur's approach

Since the development of vaccine therapy there has been an interest in the elaboration of a staphylococcal vaccine. Until the 1970s a variety of whole staphylococcal preparations was used in clinical and veterinary trials: these included cultured, attenuated, fixed or lysed organisms (Pankey, Boddie, Watts & Nickerson, 1985; Michie, 2002; García-Lara & Foster, 2009; Pellegrino, Giraudo, Raspanti, Odierno & Bogni, 2010; Pereira et al., 2011). However, little benefit and common adverse reactions were seen when using these preparations (García-Lara & Foster, 2009). In humans, staphylococci cause a wide variety of infections, including bacteraemia (Fischetti et al., 2006). One vaccine, SA75 developed by the Vaccine Research International Plc and composed of a whole cell extract against staphylococcal infections, was evaluated in a phase I clinical trial and induced a significant immune response in

healthy individuals but it is still waiting for further clinical trials (Vaccine Research International Plc website). In food-producing animals, staphylococci, namely *S. aureus*, are the main causative agent of bovine, caprine and ovine mastitis (Wilson, Gonzalez & Das, 1997). Bovine mastitis is the most prevalent disease of diary cows responsible for major economic losses on dairy farms worldwide (Wilson et al., 1997). The main causative agent of bovine mastitis is *S. aureus* (Pellegrino et al., 2010). In light of these facts some studies have addressed the efficacy of an avirulent *S. aureus* vaccine to control bovine mastitis. There are actually three commercially available inactivated *S. aureus* vaccines, Lysigin® (Boehringer Ingelheim Vetmedica, Inc., Ingelheim am Rhein, Germany), Mastivac® (Laboratorios Ovejero S.A., León, Spain) and Startvac® (Laboratorios Hipra S. A., Girona, Spain). There is also one inactivated *S. aureus* vaccine licensed for caprine and ovine mastitis, named Vimco® (Laboratorios Hipra S. A., Girona, Spain). Although a recent systematic review suggested that vaccines that employ new long-standing bacterins have achieved good results, which supports their use in the prevention and control of bovine mastitis caused by *S. aureus*, methodological differences and in some cases, a lack of more severe scientific criteria (such as double blind protocols) hinder the assessment of the effectiveness of these vaccines (Pereira et al., 2011). In small animals, like dogs and cats, staphylococci, namely *S. pseudintermedius*, are the main cause of pyoderma and otitis, infections very common in daily veterinary practice (DeBoer, Moriello, Thomas & Schultz, 1990). In the 80s another *S. aureus* vaccine combined with a phage lysate, SPL® (Delmont Laboratories Inc., Swarthmore, United States of America), was used and considered a highly effective bacterial antigen licensed for the treatment of idiopathic pyoderma caused by *S. (pseud)intermedius* in dogs (DeBoer et al., 1990). Although this vaccine is still commercialized nowadays, only a few reports have addressed the real efficacy of this bacterin/phage lysate and so it is not widely recommended for routine use (DeBoer & Marsella, 2001).

Pasteur's approach has been used also with a different objective: identification of the repertoire of antigens necessary to elicit a widespread antibody response against invasive staphylococci by immunization with inactivated attenuated strains in animal models of *S. aureus* infection, combined with high-throughput screening (Burnside, Lembo, Harrell, Klein, Lopez-Guisa, Siegesmund, Torgerson, Oukka, Molina & Rajagopal, 2012). Interestingly, the results suggested that protection against *S. aureus* infections requires antibody responses to the wide repertoire of antigens/virulence factors (Burnside et al., 2012), which is actually one of the reasons appointed for the failure of single-antigen vaccines against *S. aureus* infections (Bagnoli, Bertholet & Grandi, 2012). Yet, although strategies that incorporate whole, inactivated or attenuated *S. aureus* provide the host with an opportunity to mount a widespread antibody response, it is unlikely that incorporation of whole bacteria is an acceptable vaccine strategy (Burnside et al., 2012).

1.6.3.3.2 Second-generation approach

This approach started to be used when knowledge was improved on the pathogenesis of microbial infections, the identification of virulence factors and the characterization of the immune response after infection (Movahedi & Hampson, 2008). In fact most of the tested vaccines, including some developed against staphylococci, fall within this generation (Movahedi & Hampson, 2008; Otto, 2010). The antigens tested in these vaccines do not usually undergo a specific selection process, and are obtained from the literature based on their surface location, participation on the pathogenesis and frequency in predominant clones (Otto, 2010). One of the second-generation approaches against staphylococci includes active and passive vaccines against *S. aureus* capsular polysaccharides types 5 and 8 (for example StaphVAX™ or Altastaph™ from NABI) (Otto, 2010). These polysaccharides were selected based on good results obtained against other capsular pathogens and based on the high frequency of these capsular types in most prominent *S. aureus* clinical clones (Otto, 2010). Another approach included the use of recombinant proteins, mainly toxins like alpha-toxin and PVL (PentaStaph™ from GlaxoSmithKline), based on their major role as significant virulence factors (Otto, 2010). Recombinant vaccines targeting *S. aureus* MSCRAMMs have also been developed due to their surface location, widespread distribution and pathogenesis involvement (SA3Ag™ from Pfizer) (Otto, 2010). One other vaccine, containing a recombinant Target of RNAIII Activating Protein (rTRAP), has been tested in dairy animals (Leitner, Krifucks, Kiran & Balaban, 2011). The rTRAP vaccine was immunogenic and caused the induction of a humoral immune response, preventing new udder infections by staphylococci (Leitner et al., 2011). As TRAP is highly conserved among all strains and staphylococcal species and is constitutively expressed in any strain of *S. aureus* or CoNS, the authors considered it a universal anti-staphylococcus vaccine (Leitner et al., 2011).

Although this approach has been successfully used to select antigens against several bacterial pathogens, so far it has not been effective against staphylococci (Otto, 2010; Jansen et al., 2013). One of the factors associated with this failure is due to the staphylococci plasticity, meaning these bacteria can express several different virulence factors *in vivo* (Jansen et al., 2013). For example, staphylococci can overcome the production of capsular polysaccharides by producing proteins with the same function (Proctor, 2012). Another problem with vaccines targeting toxins, for example, is that these virulence factors are produced once the infection has been established, and so infection prevention cannot be achieved (Jansen et al., 2013). More recent approaches have been helpful in understanding the role of virulence factors *in vivo* in the staphylococcal pathogenesis and the immune response after infection (Movahedi & Hampson, 2008).

1.6.3.3.3 Third-generation approach or Reverse Vaccinology

1.6.3.3.3.1 Genomics and *in silico* prediction of antigens

A complete genome sequence can be used for several purposes. One of these purposes is the *in silico* analysis, through bioinformatics algorithms, of genes that are considered to be the most important molecules to induce a protective immune response (Movahedi & Hampson, 2008). Normally these genes of interest encode proteins or lipoproteins that are surface exposed, or secreted or are virulence factors (Movahedi & Hampson, 2008). *In silico* analysis can also be used to predict T cell and B cell epitopes, which will help optimize the best vaccine candidates (Movahedi & Hampson, 2008). The new advances in the development of bioinformatics tools coupled with faster recombination techniques and the knowledge on the host immune response will lead to new vaccines against diseases, including staphylococci (Soria-Guerra et al., 2014).

a) *In silico* analysis for detection of virulence factors

As we saw above, most of the current vaccines tested in human clinical trials so far are based on virulence factors. Several bioinformatic algorithms (Table 16) have been created to help investigators find virulence determinants based on sequence motifs (Zagursky & Russell, 2001). These bioinformatic algorithms use different models to predict the gene function, like Hidden Markov models (HMM) (Zagursky & Russell, 2001). Yet one should not forget that, ultimately, predictions need to be validated by laboratory experiments (Zagursky & Russell, 2001). One study on *S. pseudintermedius* used a combined genomic and proteomic approach to detect proteins with the LPXTG motif (Bannoehr et al., 2011). This motif is usually found in *S. aureus* cell-wall-associated surface proteins, which play important roles in virulence: ability to bind to host extracellular matrix and plasma components promoting adhesion to host tissues, evasion of host defence mechanisms and invasion of epithelial and endothelial cells (Roche, Massey, Peacock, Day, Visai, Speziale, Lam, Pallen & Foster, 2003). Besides detecting 18 proteins with the LPXTG motif in the *S. pseudintermedius* genome, the authors also identified 3 proteins containing MSCRAMMs, which represented candidate therapeutic targets for the control of bacterial pyoderma (Bannoehr et al., 2011). Another study employing a combined genomic approach was used to find motifs associated with virulence in *S. epidermidis* (Shahrooei, Hira, Khodaparast, Khodaparast, Stijlemans, Kucharíková, Burghout, Hermans & Van Eldere, 2012). After testing 5 proteins to evaluate as vaccine candidates in a mouse jugular vein catheter infection model, the authors found at least one protein was a promising target for antibody-mediated strategies against *S. epidermidis* biofilm formation (Shahrooei et al., 2012).

Table 16. Bioinformatic algorithms for prediction of virulence factors.

Name	Website	Type of information	Reference
Blast	http://blast.ncbi.nlm.nih.gov/Blast.cgi	Compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches	Altschul et al., 1990
ScanProsite	http://prosite.expasy.org/scanprosite/	Scans for protein domains, families and functional sites as well as associated patterns and profiles	Sigrist et al., 2002; De Castro et al., 2006
InterProScan	http://www.ebi.ac.uk/interpro/interproscan.html/	Provides functional analysis of proteins by classifying them into families and predicting domains and important sites	Jones et al., 2014; Mitchell et al., 2015
Pfam	http://pfam.xfam.org/	Analyses your protein sequence for Pfam matches	Finn et al., 2014
ProDom	http://prodom.prabi.fr/prodom/current/html/home.php	Builds multiple alignments, phylogenetic trees and domain architectures of proteins, as well as a BLAST-based server to analyse new sequences for homologous domains	Servant et al., 2002; Bru et al., 2005
BLAST PRINTS	http://www.bioinf.manchester.ac.uk/cgi-bin/dbbrowser/PRINTS/printsBLAST.cgi	Gives the familiar form of output, but modified by means f direct links both to the familial discriminators in PRINTS and fingerprint profile visualization software	Attwood et al., 1999; Wright et al., 1999
SMART	http://smart.embl-heidelberg.de	Identificaties and annotates signalling domain sequences	Schultz et al., 1998
HAMAP-Scan	http://hamap.expasy.org/hamap_scan.html	Uses manually built annotation templates for protein families to propagate annotation to all members of manually defined protein families, using very strict criteria	Lima et al., 2009
CATCH	http://www.cathdb.info	Groups protein domains into superfamilies when there is sufficient evidence they have diverged from a common ancestor	Sillitoe et al., 2013
SUPERFAMILY	http://supfam.cs.bris.ac.uk/SUPERFAMILY/	Provides sequencing searching, multiple alignments to sequences of known structure, and structural assignments to all complete genomes	Gough & Chothia, 2002
PIRSFM	http://pir.georgetown.edu/pirwww/dbinfo/pirsf.shtml	Annotates both specific biological and generic biochemical func-	Wu et al., 2004

		tions	
PANTHER	http://www.pantherdb.org	Relates protein sequence relationships to function relationships in a robust and accurate way	Thomas et al., 2003
TIGRFAMs	http://www.jcvi.org/cgi-bin/tigrfams/index.cgi	Provides curated multiple sequence alignments, HMM for protein sequence classification, and associated information designed to support automated annotation of proteins	Haft et al., 2001
ClustalOmega	http://www.ebi.ac.uk/Tools/msa/clustalo/	Performs multiple sequence alignment program that uses seeded guide trees and HMM profile-profile techniques to generate align-ments between three or more sequence	Sievers et al., 2011

b) *In silico* analysis for detection of surface-exposed proteins

Another approach that has been used frequently in combination with the previous methodology is the identification of surface-exposed proteins. In fact, for vaccine development, it has been previously suggested that more importantly than finding a gene function, is to find out if the gene encodes a surface-exposed antigen (Zagursky & Russell, 2001). In the course of an infection, the bacterial outer cell membrane components, as well as secreted proteins, represent the interphase of the bacterium-host interaction and are exposed to the host immune system (Chakravarti, Fiske, Fletcher & Zagursky, 2000; Chitlaru, Gat, Grosfeld, Inbar, Gozlan & Shafferman, 2007). Several bioinformatic algorithms have been developed with this purpose and some are shown in Table 17. These algorithms employ different models, to detect, for example, transmembrane helices or signal peptides (Movahedi & Hampson, 2008). The two studies presented above (Bannoehr et al., 2011; Shahrooei et al., 2012), also used algorithms to predict surface location. In the *S. pseudintermedius* genome, 60 proteins and in the *S. epidermidis* genome 64 proteins were possibly surface-localized (Bannoehr et al., 2011; Shahrooei et al., 2012). Surface-exposed proteins are favoured vaccine candidates since they are more easily accessible microbial antigens to the host's immune system (Zagursky & Russell, 2001).

c) *In silico* analysis for prediction of B cells and T cells epitopes

In order for an antigen to be effective, it must elicit a T cell and a B cell response (Davies & Flower, 2007). Newly created *in silico* algorithms and databases can be used to identify, characterize or predict antigen epitopes recognized by T- and B-lymphocytes (Bambini & Rappuoli, 2009). These algorithms are able to recognize peptide fragments of pathogen antigens exhibited by MHC proteins at the surface of antigen-presenting cells (Bambini & Rappuoli, 2009). B-cell epitopes are defined by the discrete surface region of an antigen bound by the variable domain of an antibody (Bambini & Rappuoli, 2009). While T-cell epitopes are short linear peptides, B-cell epitopes can be linear contiguous amino acids or they can be discontinuous amino acids, separated within the sequence but brought together in the folded protein, known as conformational epitopes (Bambini & Rappuoli, 2009). Several algorithms have been developed so far, from which some are described in Table 18.

One study used this strategy to design T-cell epitope candidates against *S. aureus* endocarditis in humans (Oprea & Antohe, 2013). By selecting and performing structural analysis, antigenicity testing and identification of B cell and T cell epitopes on 10 proteins, previously identified as surface-exposed, the authors were able to identify five T-cell epitopes that could potentially be used in a vaccine (Oprea & Antohe, 2013).

Table 17. Bioinformatic algorithms for prediction of subcellular location.

Name	Website	Type of information	Reference
PSORTb	http://www.psort.org/psortb/	Consists of multiple analytical modules, each of which analyses one biological feature known to influence or be characteristic of subcellular localization	Yu et al., 2010
ProtLock	http://bioinf.uab.es/cgi-bin/trsdb/protloc.cgi	Assigns a possible cellular localization of polypeptide sequences according to their amino acid frequencies against predefined sets	Cedano et al., 1997
SignalP	http://www.cbs.dtu.dk/services/SignalP/	Predicts the presence and location of signal peptide cleavage sites in amino acid sequences from different organisms	Petersen et al., 2011
SecretomeP	http://www.cbs.dtu.dk/services/SecretomeP/	Produces *ab initio* predictions of non-classical i.e. not signal peptide triggered protein secretion	Bendtsen et al., 2006
TatP	http://www.cbs.dtu.dk/services/TatP/	Predicts the presence and location of Twin-arginine signal peptide cleavage sites in bacteria	Bendtsen et al., 2005
TMHMM	http://www.cbs.dtu.dk/services/TMHMM/	Predicts of transmembrane helices in proteins	Krogh et al., 2001
TopPred	http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::toppred	Predicts membrane proteins	von Heijne, 1992
PRED-TMR	http://athina.biol.uoa.gr/PRED-TMR/	Predicts transmembrane domains in proteins using solely information contained in the sequence itself	Pasquier et al., 1999
DAS	http://www.sbc.su.se/~miklos/DAS/	Predicts transmembrane regions of a query sequence	Cserzo et al., 1997
PredictProtein	https://www.predictprotein.org	Predicts secondary structure and returning families of related proteins	Yachdav et al., 2014

Table 18. Bioinformatic algorithms for prediction of B cells and T cells epitopes.

Name	Website	Type of information	Reference
BepiPred	http://www.cbs.dtu.dk/services/BepiPred/	Predicts the location of linear B-cell epitopes using a combination of a hidden Markov model and a propensity scale method	Larsen et al., 2006
DiscoTope	http://www.cbs.dtu.dk/services/DiscoTope/	Predicts discontinuous B cell epitopes from protein three dimensional structures	Kringelum et al., 2012
NetMHCpan	http://www.cbs.dtu.dk/services/NetMHCpan/	Predicts binding of peptides to any known MHC molecule using artificial neural networks	Hoof et al., 2009
NetMHCcons	http://www.cbs.dtu.dk/services/NetMHCcons/	Predicts binding of peptides to any known MHC class I molecule	Karosiene et al., 2012

More recently, in another study three important virulence factors of *S. aureus* were selected, clumping factor A (ClfA), iron-regulated surface determinant (IsdB), and gamma hemolysin (Hlg) to form a chimeric protein and bioinformatic tools were used to predict the chimeric protein antigenicity and linear and conformational B-cell epitopes (Delfani, Fooladi, Mobarez, Emaneini, Amani & Sedighian, 2015). The data indicated that epitopes of the chimeric protein, designed from ClfA, IsdB, and Hlg of *S. aureus* could induce B-cell–mediated immune responses successfully and therefore, could be used as a vaccine candidate against *S. aureus* infections (Delfani et al., 2015).

One of the limitations of this analysis is the lack of accuracy, although T-cell epitopes are more accurate and comprehensive than B-cell epitopes (Movahedi & Hampson, 2008). Another limitation is that most algorithms so far developed only predict T-cell epitopes for humans and a few animals (Movahedi & Hampson, 2008). Finally, although this approach saves the investigators time before going to the laboratory, all the identified epitopes require subsequent experimental validation in order to ascertain the suitability of the epitopes for vaccine development (Oprea & Antohe, 2013).

1.6.3.3.3.2 Transcriptomics

By analysing the changes that occur in the bacterial gene expression during infection investigators are able to understand which determinants are essential for bacterial pathogenesis and for bacterial survival (Kaushik & Sehgal, 2008). This analysis can be done at the level of the complete genome, for example by using complementary deoxyribonucleic acid (cDNA) microarrays, by the newly technique RNA-seq, or by targeting the expression of specific genes involved, for example in the pathogenesis of infection, by qRT-PCR (Dhiman, Bonilla, O'Kane & Poland, 2001).

Transcriptomics has been used to detect changes in the expression of particular staphylococcal genes. In one study, the investigators were interested in the expression of *sdrG*, a gene encoding serine-aspartate repeat-containing protein G, present in most strains of *S. epidermidis* (Sellman, Timofeyeva, Nanra, Scott, Fulginiti, Matsuka & Baker, 2008). This gene was expressed early during infection in response to specific host environmental cues present in the bloodstream and resulted in a concomitant increase in SdrG protein levels (Sellman et al., 2008). The authors concluded that SdrG possessed attributes of a vaccine component effective against the pathogenic form of *S. epidermidis* (Sellman et al., 2008). In another study, the expression of the *fhuD2* gene, encoding the ferric hydroxamate-binding lipoprotein, was evaluated in a murine renal abscess model of infection (Mishra, Mariotti, Fiaschi, Nosari, Maccari, Liberatori, Fontana, Pezzicoli, De Falco, Falugi, Altindis, Serruto, Grandi & Bagnoli, 2012). Using qRT-PCR, the authors detected up-regulation of the *fhuD2* gene in bacteria recovered from infected animals, which was accompanied by up-regulation of the

FhuD2 protein in infected tissues and was required for staphylococcal dissemination and abscess formation suggesting it could be efficacious as a vaccine target (Mishra et al. 2012).
One of the limitations of this technique is the amount of RNA needed for the experiments, especially for the microarray analysis, which is not always possible when performing *in vivo* studies (Kaushik & Sehgal, 2008). However, new techniques like RNA-seq are overcoming this problem, and are able to do massively parallel sequencing of RNA (or, in fact, the corresponding cDNA), based on next-generation sequencing (NGS) platforms (Westermann, Gorski & Vogel, 2012). Apart from also being able to quantify the RNA expression like microarrays, RNA-seq provides possibility for the simultaneously determination of the host and pathogen transcriptomes providing the potential to further get insights into the host-pathogen interaction (Westermann et al., 2012). Still, another major limitation that cannot be undergone by any of these techniques, is the simple fact that there is not a direct correlation between mRNA and protein expression level (Bambini & Rappuoli, 2009). In this way, higher levels of mRNA expression do not necessarily mean higher levels of the corresponding protein and further studies are needed to confirm the hypothesis (Bambini & Rappuoli, 2009).

1.6.3.3.3.3 Proteomics

Genomics and transcriptomics can give fundamental information on the potential antigens produced by a bacterial pathogen; however only proteomics gives evidence on the protein expression during the bacteria's life and during host-pathogen interactions (Serruto & Rappuoli, 2006). Proteomic analysis involves protein digestion, eventually preceded by its separation using one-dimension electrophoresis (1-DE) or two-dimension electrophoresis (2-DE) followed by liquid chromatography coupled to mass spectrometry/mass spectrometry (LC)-MS/MS or matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS), respectively (Nandakumar, Nandakumar, Marten & Ross, 2005; Resch, Leicht, Saric, Pásztor, Jakob, Götz & Nordheim, 2006; Planchon, Chambon, Desvaux, Chafsey, Leroy, Talon & Hébraud, 2007). Proteomics allows the identification of post-translational modifications, differentially expressed proteins and protein-protein interactions (Movahedi & Hampson, 2008). Like for genomics, in the proteomic approach to bacterial vaccine development preference is given to surface proteins (Serruto & Rappuoli, 2006; Movahedi & Hampson, 2008).
This approach has been used extensively to study different staphylococci in different growth conditions, including during biofilm formation (Kohler, Wolff, Albrecht, Fuchs, Becher, Büttner, Engelmann & Hecker, 2005; Nandakumar et al., 2005; Resch et al., 2006; Planchon et al., 2007; Bannoehr et al., 2011). Other studies identified proteins differentially produced during infection, like mastitis (Le Maréchal, Jardin, Briard-Bion, Rault, Berkova, Vautor, Thiéry, Even & Le Loir, 2013), while in others the investigators compared the proteomes of invasive and commensal staphylococcal strains (Yang, Li, Chen, Ou, Jin, Lu, Zhu, Qin, Qu &

Yang, 2006; Seyffert, Le Maréchal, Jardin, McCulloch, Rosado, Miyoshi, Even, Jan, Berkova, Vautor, Thiéry, Azevedo & Le Loir, 2012).

One of the limitations of this approach is the large amount of proteins that are produced under *in vitro* conditions that may not correspond to *in vivo* infection (Movahedi & Hampson, 2008). This can be overcome by using models of *in vivo* infection, but this may not always be easy, especially in human infections (Movahedi & Hampson, 2008). Another limitation of proteomics is that some proteins are poorly resolved by 2-DE, like membrane-anchored proteins due to its hydrophobicity (Wilkins, Gasteiger, Sanchez, Barioch & Hochstrasser, 1998; Nandakumar et al., 2005) or low abundance proteins (Bröker & van Belkum, 2011). This has been recently overcome by the GeLC-MS/MS approach, which does not rely on 2-DE separation (Chandramouli & Qian, 2009). More recently, shotgun approaches, which do not involve any protein separation, have been developed and present a high-resolution separation of peptide digests (Chandramouli & Qian, 2009). Yet, this type of assays requires very expensive equipment and highly specialized apparatus (Chandramouli & Qian, 2009). The last limitation is that proteomics alone cannot identify the potential immunogenicity of a protein and so it has been combined with serological analysis (Movahedi & Hampson, 2008).

1.6.3.3.3.4 Serological proteomics

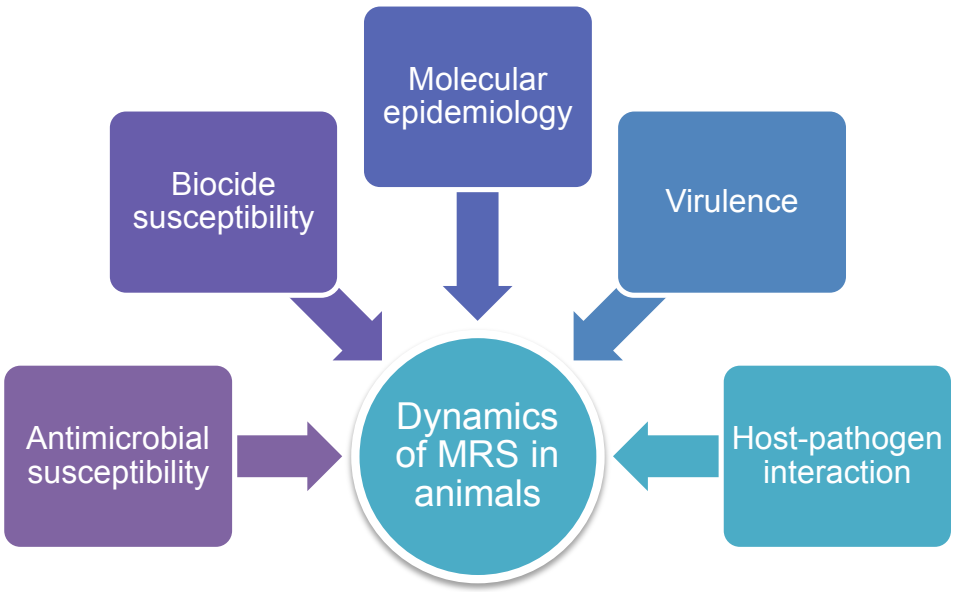
Serological proteomics, also called Immunoproteomics, is a technique that combines 2-DE, 2-DE western blotting (also called immunoblotting) and antigens identification through MS (Klade, Voss, Krystek, Ahorn, Zatloukal, Pummer & Günther, 2001; Chitlaru et al., 2007). In this approach, the immunogenic proteins are identified using sera with antistaphylococcal antibodies from healthy individuals and infected patients (Bröker & van Belkum, 2011). The protocol involves protein extraction of the microorganism of interest and subsequent protein separation by 2-DE (Bröker & van Belkum, 2011). The proteins are then transferred to a membrane and incubated with serum antibodies (Bröker & van Belkum, 2011). Following incubation with a secondary antibody, the highly immunogenic proteins are visualized and matched with spots in the protein gels. The gel spots are then excised and identified by MS (Bröker & van Belkum, 2011). However, there are some limitations in serological proteomics. First of all, only proteins resolved by 2-DE can be subsequently identified by serum antibodies (Bröker & van Belkum, 2011). However, as discussed above, in the 2-DE method several proteins may not be separated (Wilkins et al., 1998; Nandakumar et al., 2005) or low abundance proteins may not be detected (Bröker & van Belkum, 2011). Furthermore, the *in vitro* conditions used (like bacterial growth) may not mimic the *in vivo* protein expression and so not all the relevant proteins may be present (Bröker & van Belkum, 2011). Another problem that may occur is that antibody binding cannot always be attributed to a single protein but to a group of proteins (Bröker & van Belkum, 2011). Matching the proteins in the gel and in the membrane may not be very easy either and since immunoblotting is more sensitive than the

staining for general protein detection, antibody binding to low abundance proteins may erroneously be attributed to a major spot in its vicinity (Bröker & van Belkum, 2011).
In spite of its limitations, this methodology has been used to identify staphylococcal antigenic proteins. Vytvytska and colleagues performed the first work in *S. aureus* (2002). The authors used a *S. aureus* laboratory strain and two pools of selected human sera (healthy donors and patients) to identify highly immunogenic proteins (Vytvytska, Nagy, Blüggel, Meyer, Kurzbauer, Huber & Klade, 2002). The authors were able to identify very strong immunoglobulin G (IgG)-reactivity against multiple proteins using both pooled sera from healthy individuals and patients (Vytvytska et al., 2002). This led to the identification of 15 proteins including novel and known vaccine candidates (Vytvytska et al., 2002). Other studies have been described after, including serological approaches with sera from ruminants with mastitis (Tedeschi, Taverna, Negri, Piccinini, Nonnis, Ronchi & Zecconi, 2009; Le Maréchal, Jan, Even, McCulloch, Azevedo, Thiéry, Vautor & Le Loir, 2009)

1.6.3.3.3.5 Antigenomics

This approach combines the advantages of full genome coverage and serological antigen identification (Meinke, Henics, Hanner, Minh & Nagy, 2005). It is similar to the Serological Proteomics approach, and the difference relies in the “protein library” preparation. In this method, the selection of vaccine candidates, either small linear epitopes or medium-sized conformational epitopes encoded by the bacterial genome are displayed on the surface of *Escherichia coli* via outer membrane proteins (Henics, Winkler, Pfeifer, Gill, Buschle, von Gabain & Meinke, 2003). The antigenome technology does not rely on genome annotation and, thus, has the potential to select proteins that are not predicted by ORF-finding algorithms and does not have the problem of *in vitro* vs *in vivo* protein expression (Meinke, Henics, Hanner, Minh & Nagy, 2005). This technology has been used to identify staphylococcal antigens (Etz, Minh, Henics, Dryla, Winkler, Triska, Boyd, Söllner, Schmidt, von Ahsen, Buschle, Gill, Kolonay, Khalak, Fraser, von Gabain, Nagy & Meinke, 2002; Weichhart, Horky, Söllner, Gangl, Henics, Nagy, Meinke, von Gabain, Fraser, Gill, Hafner, & von Ahsen, 2003; Dryla et al., 2005). In one of these studies, the authors determined a total of 60 antigenic proteins based on their reactivity with individual sera from patients and healthy individuals (Etz et al., 2002). This study was actually one of the two studies that identified the iron-regulated surface determinant B (IsdB) protein as an antigen and was used in vaccine V710™ from Merck (Kuklin, Clark, Secore, Cook, Cope, McNeely, Noble, Brown, Zorman, Wang, Pancari, Fan, Isett, Burgess, Bryan, Brownlow, George, Meinz, Liddell, Kelly, Schultz, Montgomery, Onishi, Losada, Martin, Ebert, Tan, Schofield, Nagy, Meineke, Joyce, Kurtz, Caulfield, Jansen, McClements & Anderson, 2006; Harro, Betts, Orenstein, Kwak, Greenberg, Onorato, Hartzel, Lipka, DiNubile & Kartsonis, 2010). The authors considered the approach as having the potential to greatly accelerate and facilitate the formulation of novel vaccines (Etz et al.,

2002) and this approach has been used to identify vaccine candidate antigens from other bacterial species. However, one major limitation of this technique is that it can only detect linear contiguous epitopes, because conformational epitopes that are assembled as a result of protein folding may not be detected (Kaushik & Sehgal, 2008).



2 Objectives

The importance of MRS has been recognized in animals and knowledge on the epidemiology of MRS is important since these strains pose a serious public health threat. Furthermore, the antimicrobial and biocide susceptibility and the molecular epidemiology of these strains can contribute to monitor the spread of antimicrobial/biocide resistance genes/strains and consequently control its dissemination. In the first part of the study, we determined the frequency and characteristics of MRS isolated from different animal species. In a second part of this work, a focus was given to *S. pseudintermedius* since this bacterium has become a serious veterinary problem due to its multidrug resistant profile. In this way, novel strategies are needed for the treatment of *S. pseudintermedius* infections and more information on the interaction of this pathogen with its host is indispensable.

The main objectives and approaches of this study were:

I. Determine the frequency of MRSA strains in several animal species, identify the characteristics of MRSA present in animals and compare to human strains

a) Determine the frequency and characteristics of MRSA colonization in horses and calves

b) Characterize the clonal diversity, antimicrobial/biocide susceptibility patterns, resistance genes and virulence factors of MRSA from animals, environment and humans in contact with animals

II. Reveal antimicrobial/biocide susceptibility patterns/trends and resistance genes in MRS strains

a) Identify the antimicrobial/biocide susceptibility and resistance genes in MRS strains from horses

b) Compare the antimicrobial/biocide susceptibility and resistance genes between MRSP and MSSP strains

c) Detect the trends of antimicrobial resistance, resistance mechanisms and molecular characteristics of MRS in companion animals

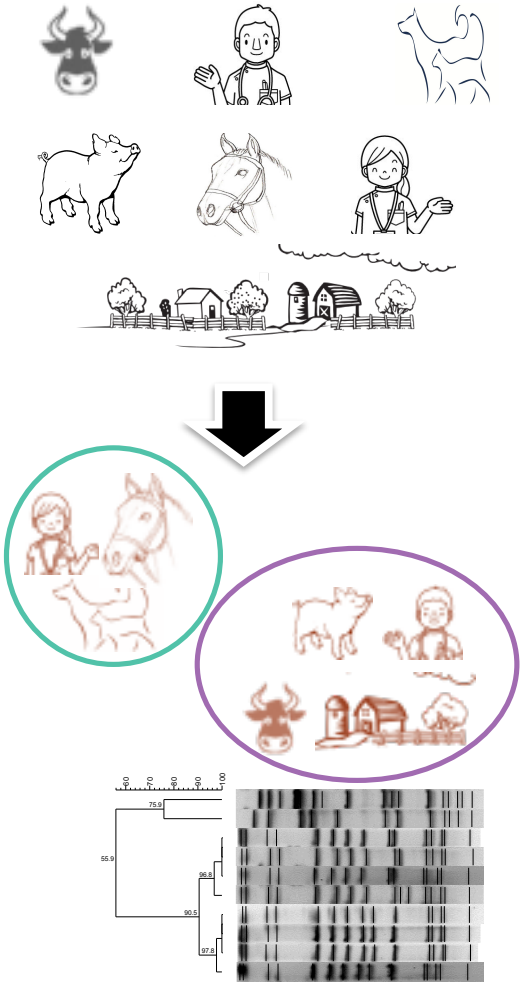
III. Study the pathogenesis of *Staphylococcus pseudintermedius* infections in dogs

a) Identify differences in the expression of virulence factors and regulatory systems between MRSP and MSSP

b) Characterize the *S. pseudintermedius* proteome and identify antigenic proteins

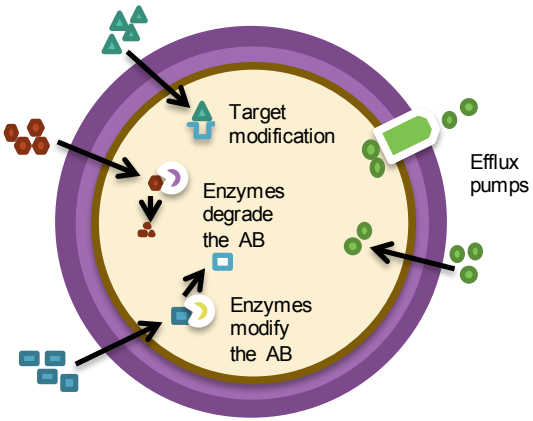
Part 1

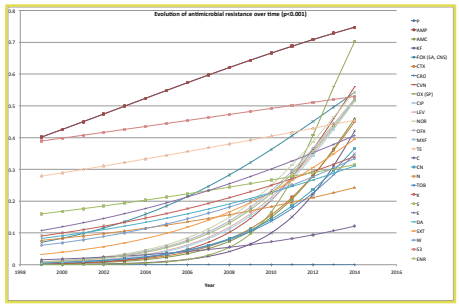
Epidemiology of MRSA



Part 2

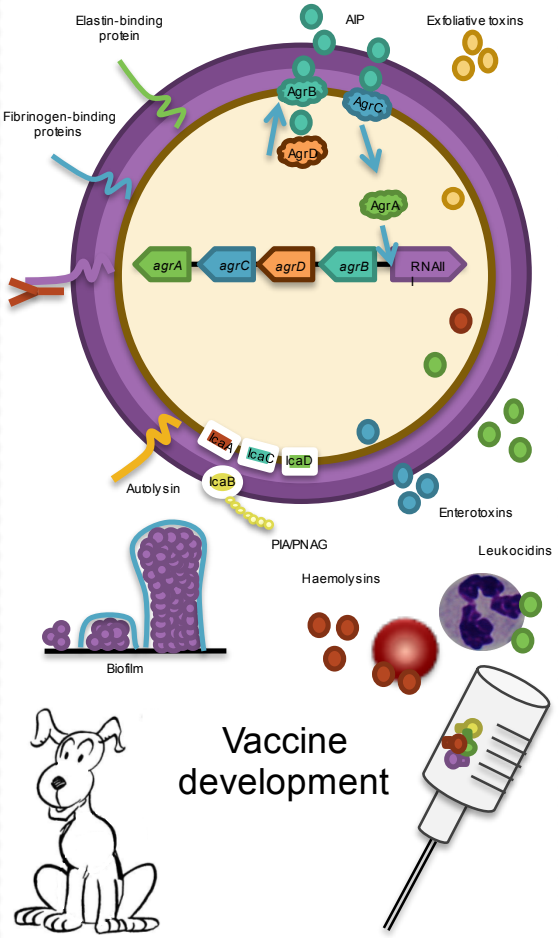
Antimicrobial and biocide susceptibility of MRS

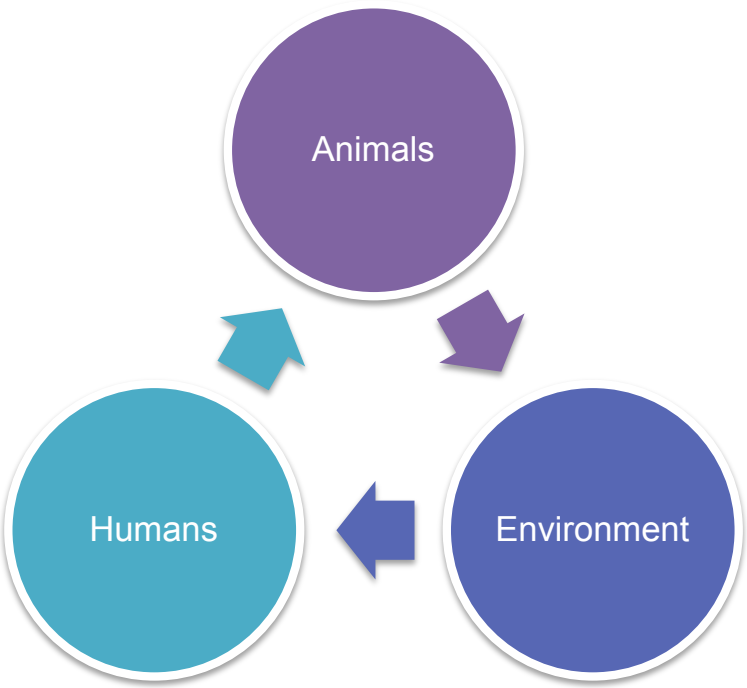




Part 3

Pathogenesis of *S. pseudintermedius*





3 The study

3.1 Part 1 – Epidemiology of methicillin-resistant *Staphylococcus aureus* in animals in Portugal

3.1.1 First Report of Methicillin-Resistant *Staphylococcus aureus* ST5 and ST398 from Purebred Lusitano Horses

Paper published in *Journal of Equine Veterinary Science*

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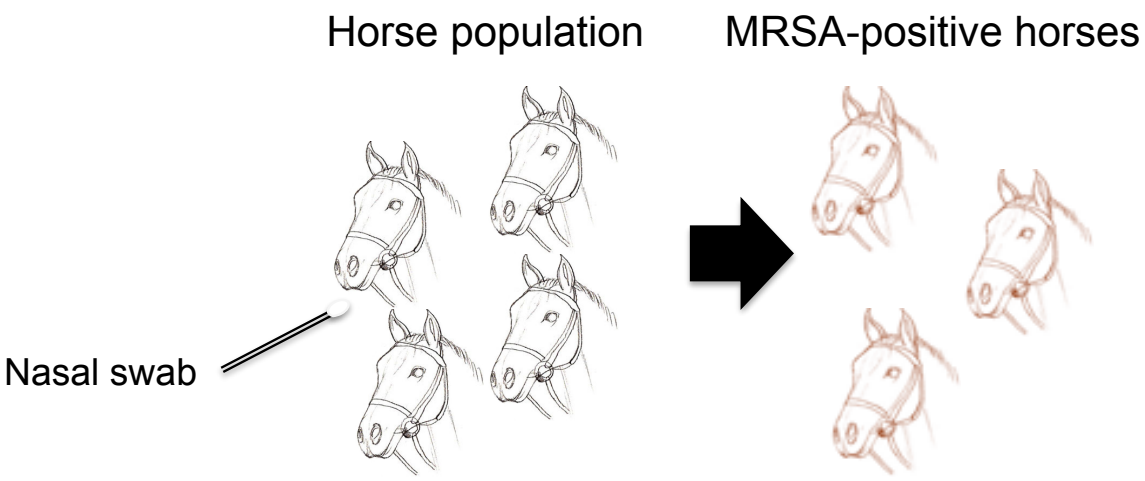

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Short Communication

First Report of Methicillin-Resistant *Staphylococcus aureus* ST5 and ST398 from Purebred Lusitano Horses

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ABSTRACT

Methicillin-resistant *Staphylococcus aureus* (MRSA) was first described in horses in 1996. The frequency of MRSA colonization in horses varies among European countries, but it is unknown in Portugal. The aim of this study was to screen for MRSA nasal carriage in a sample of horses entering the Equine Unit, Large Animal Veterinary Teaching Hospital of the Faculty of Veterinary Medicine, Lisbon, Portugal. Seventy-one horses were swabbed, and MRSA was identified by selective isolation on a chromogenic medium. Two *S aureus* isolates showed resistance to oxacillin (minimum inhibitory concentration >4 μg/mL) and contained the *mecA* gene. Both strains were isolated from purebred Lusitano horses that lived in farms with more than 20 equines. These MRSA strains represented two different clones: isolate FMVA3/10 was an MRSA sequence type ST5 with a staphylococcal cassette chromosome *mec* VI, coresistant to erythromycin and clindamycin; and isolate FMVA16/10 was sequence type ST398, with a staphylococcal cassette chromosome *mec* IV, coresistant to tetracycline, gentamicin, and trimethoprim. Isolate FMVA3/10 represents a human epidemic clone not previously reported among horses in Europe, which once again reinforces the fact that transmission of MRSA clones between horses and humans occurs. Isolate FMVA16/10 represents the first report of the detection of MRSA ST398 among horses in Portugal. Lusitano horses can carry animal and human MRSA in the nostrils, acting as reservoirs, which can potentially be transmitted to humans.

1. Introduction

Methicillin-resistant *S aureus* (MRSA) was first reported in horses in 1996, as a cause of metritis in Japan [1]. Since then, several other reports have described MRSA as a cause of a variety of other infections, including several life-threatening conditions such as pneumonia and bloodstream infection [2]. The prevalence of MRSA colonization has been studied in numerous horse populations in various countries, with rates ranging from 0% to 10.9% [2]. Still, in Portugal, there are no reports of equine MRSA infections, and the rate of MRSA carriage in horses is unknown. Portugal is one of the countries in Europe with the highest prevalence of nosocomial invasive MRSA isolates in humans [3], and a recent report described a prevalence of MRSA carriage of 1.4% in cats and 0.7% in dogs [4].

The first reports of MRSA in horses described sequence type (ST) 8 (USA500 or CMRSA-5) as the major clone predominant in this species in the North American continent [2]. MRSA ST8 was first described in humans but accounts only for a small percentage of human infections [2].

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Table 1
Clinical epidemiology and pathology characterization of horses enrolled in the study

Study Group	Age Range (Years)	Gender	Breed	Geographical Origin	Portugal Region	Pathology Characterization
Recurrent airway obstruction (RAO) horses (n = 30)	7-30	Mare−8	Purebred Lusitano−16	Rural−19	Estremadura−22	RAO stages:
		Gelding−6	Lusitano cross−14	City−6	Ribatejo−8	Stage 1-4 horses
		Stallion−16		Town−1		Stage 2-7 horses
				Village−4		Stage 3-10 horses
						Stage 4-9 horses
Other respiratory pathology cases (n = 8)	2-20	Mare−2	Purebred Lusitano−3	Rural−4	Estremadura−7	Dorsal displacement of the soft palate (DDSP)−3
		Gelding−2	Lusitano cross−5	City−1	Ribatejo−1	4th brachial arch defects (4-BAD)−1
		Stallion−4		Town−2		Grade 4 right recurrent laryngeal neuropathy (RLN)−1
						Grade 3 left RLN−2
				Village−1		Pneumonia−1
Equine gastric ulcer syndrome (EGUS) horses (n = 13)	7-20	Mare−0	Purebred Lusitano−13	Rural−0	Estremadura−13	Number of lesions (a):
		Gelding−0		City−0	Ribatejo−0	Grade 0-0
		Stallion−13		Town−13		Grade 1-1
				Village−0		Grade 2-1
						Grade 3-2
						Grade 4-8
						Severity of lesions (b):
						Grade 0-0
						Grade 1-2
						Grade 2-1
						Grade 3-3
						Grade 4-0
						Grade 5-6
Control group (n = 20)	0.2-22	Mare−7	Purebred Lusitano−9	Rural−8	Estremadura−12	Control group of a RAO study −10
		Gelding−3	Lusitano cross−11	City−8	Ribatejo−8	Elective surgery−9 (1 Corneal laceration; 3 Standard castrations; 2 Cryptorchid castrations; 1 Calcinosis circumscripta; 1 Umbilical hernia; 1 Dental fistula)
		Stallion−10		Town−2		
				Village−2		
						Hyperlipemia-1

(a) Classification according to the number of lesions (MacAllister et al., 1997): grade 0−without lesions; grade 1−1-2 lesions; grade 2−3-5 lesions; grade 3−6-10 lesions; grade 4 to >10 lesions or diffuse lesions or of large dimensions.
(b) Classification according to severity of lesions (MacAllister et al., 1997): grade 0−without lesions; grade 1−superficial lesions only in the mucosa (pink and without raised borders); grade 2−deeper lesions than in grade 1, including deeper structures (pink crater with raised borders); grade 3−multiple lesions of varying severity (grade 1 and 2 and at least one grade 4 lesion); grade 4−lesions, including deeper structures and with an active appearance (hyperemic, darkened, or necrotic crater); grade 5−Lesion severity similar to grade 4 but with active hemorrhage or with adherent blood clot.

This led to the suggestion that this human epidemic clone could have adapted to horses [2]. In Europe, however, ST398 is the main clone associated with horses both as a cause of infection and colonization [1]. Weese and van Duijkeren proposed that this clone could have entered the horse population from food animals, directly or indirectly [2].

Owing to the lack of knowledge on MRSA in horses in Portugal, the aim of this study was to screen a population of horses admitted to a Veterinary Hospital to determine the MRSA colonization frequency and the type of MRSA strains involved. This could be a first estimation of the equine MRSA colonization status in a country with a high prevalence of human hospital-acquired MRSA infection.

2. Methods

2.1. Study Population

Between March 2008 and October 2010, a total 71 horses entering the Equine Unit, Large Animal Veterinary Teaching Hospital of the Faculty of Veterinary Medicine−Technical University of Lisbon, were enrolled in the study. All horses from the facility, which stayed in the hospital for at least one night, were enrolled after owner's consent. Thirty horses had recurrent airway obstruction (RAO); eight had a respiratory pathology, other than RAO; 13 had equine gastrointestinal ulcer syndrome (EGUS), and 20 were healthy horses that were admitted to either elective surgery (castration) or as part of a control group for an RAO study (Table 1). The horses' gender, age range, breed, stable geographic localization, and type are summarized in Table 1. This table also contains staging and characterization of the equines' diseases. Horses presenting with RAO were classified into the following stages: stage 0−No RAO; stage 1−latent RAO; stage 2−mild RAO; stage 3−moderate RAO; stage 4−severe RAO. The horses were classified on the basis of the following parameters: clinical evaluation of cough, nostril flare, and abdominal lift; endoscopic evaluation of airway mucus accumulation, apparent viscosity, localization, and color; X-ray evaluation of interstitial pattern, bronchial radiopacity, bronchial, and tracheal thickening; and bronchoalveolar lavage fluid cytology neutrophil percentage. Horses with EGUS were classified according to the number and severity of the lesions (see Table 1) as previously described [5].

Table 2
Molecular characteristics and antimicrobial resistance profiles of methicillin-resistant *Staphylococcus aureus* isolated from purebred Lusitano horses presented at the Equine Unit, Large Animal Veterinary Teaching Hospital of the Faculty of Veterinary Medicine, Technical University of Lisbon, Portugal

Strain	Origin	Antimicrobial-Associated Coresistance Patterns	Antimicrobial-Associated Coresistance Genes	SCC*mec* Type	*spa* Type	ST Type
FMVA3/10	Horse with EGUS	FA^r, E^r, CLI^r	*fusC*, *ermC*	VI	t062	5
FMVA16/10	Healthy horse	TET^r, GEN^r, W^r	*tetM*, *aac(6')-Ie-aph(2'')-Ia*, *dfrK*	IV	t011	398

CLI, clindamycin; E, erythromycin; EGUS, equine gastric ulcer syndrome; FA, fusidic acid; GEN, gentamicin; SCC*mec*, staphylococcal cassette chromosome *mec*; ST, sequence type; W, trimethoprim.

2.2. Bacterial Isolates

All 71 horses were nasally swabbed after admission to the hospital. Samples were collected by inserting a cotton swab approximately 10 cm into one anterior nostril, and then rolling it while removing. The swabs were placed in modified Amies medium and stored at 4°C until processing. Swabs were first enriched in 3 mL of Mueller–Hinton broth containing 65 g/L NaCl for 24 hours at 37°C, and then 500 μL were inoculated into 3-mL tryptone soy broth with 3.5 mg/L cefoxitin and 75 mg/L aztreonam. After incubation for 24 hours at 37°C, a 10-μL loopful was then plated onto a chromogenic agar selective for MRSA, ChromID MRSA (bioMérieux, Marcy L'Etoile, France). Incubation was carried out for 24 and 48 hours at 37°C, as recommended by the manufacturer. On evaluation of the growth on the selective medium after 24 and 48 hours, MRSA-suspicious colonies were purified on Columbia agar plates containing 5% sheep blood (bioMérieux) for 24 hours at 37°C. Isolates were then subjected to PCR amplification of the *mecA* and *S aureus* specific-*nuc* genes [6].

2.3. Antimicrobial Susceptibility Testing

Susceptibility to oxacillin, erythromycin, tetracycline, gentamicin, clindamycin, vancomycin, enrofloxacin, fusidic acid, and trimethoprim was tested by broth microdilution using customized plates (Sensititre CMV1AMAF and BOPO6F microplates, Trek Diagnostic Systems, West Sussex, United Kingdom) and using an in-house method (trimethoprim and fusidic acid). Clinical and Laboratory Standards Institute guidelines and breakpoints were used [7,8].

2.4. Molecular Typing

Genomic DNA was isolated by the boiling method. Briefly, a loop full of bacteria was picked from a plate and transferred to phosphate-buffered saline. The suspension was centrifuged, the supernatant discarded, and the pellet resuspended in TE (Tris-EDTA buffer pH 8.0). After boiling, it was transferred directly to ice and finally diluted in TE. The antibiotic resistance genes *ermA*, *ermC*, *dfrK*, *tetK*, *tetM*, *fusB*, *fusC*, and *aac(6')-Ie-aph(2'')-Ia* were detected by PCR as described before [9,10]. Isolates were subjected to *spa* typing, SCC*mec* typing, and ST398 specific-PCR amplification [11]. They were also tested for the presence of the Panton-Valentine leukocidin (PVL) genes by PCR [12].

3. Results

Of the 71 horses swabbed, 17 were mares (24%), 11 were geldings (15%), and 43 were stallions (61%). More than half were purebred Lusitano horses (58%), whereas the remaining were Lusitano cross horses (42%). Furthermore, a high proportion of the equines were from a rural area (44%), followed by horses living in towns (25%), in cities (21%) and finally in villages (10%). The stables were distributed in two Portuguese regions, Estremadura (76%) and Ribatejo (24%), located in the central part of the country, not too far from each other.

MRSA was isolated from two purebred Lusitano horses: one horse with EGUS (8%) and one healthy horse (5%). Both MRSA isolates had minimum inhibitory concentration (MIC) of >4 μg/mL and contained the *mecA* gene. The overall MRSA colonization frequency was 3%. One MRSA isolate (FMVA3/10) was *spa* type t062, which is known to be ST5, harbored a SCC*mec* VI and was PVL-negative. Isolate FMVA3/10 was coresistant to fusidic acid (MIC 24 μg/mL), erythromycin (MIC >4 μg/mL), and clindamycin (MIC >16 μg/mL) and was positive for the antimicrobial resistance genes *fusC* and *ermC* (Table 2). This isolate can be related to two human epidemic clones: the Pediatric clone (ST5-IV/VI) and the New York/Japan clone (ST5-II). The MRSA ST5-IV clone has been described in horses, cats, dogs, and pigs in Canada (named CMRSA-2), where it is also the most common community-associated MRSA strain in humans [13]. In contrast, MRSA ST5-II has never been described in horses, although it has been reported in dogs and cats in a study conducted in the upper Midwestern and Northeastern United States [14]. Still, it is important to notice that none of these clones has ever been isolated in equine samples in Europe.

The second MRSA isolate (FMVA16/10) was *spa* type t011, ST398-PCR positive, harbored a SCC*mec* IV, and was PVL-negative. Isolate FMVA16/10 was coresistant to tetracycline (MIC >8 μg/mL), gentamicin (MIC >16 μg/mL), and trimethoprim (MIC >256 μg/mL) and was positive for the antimicrobial resistance genes *tetM*, *aac(6')-Ie-aph(2'')-Ia*, and *dfrK* (Table 2). This is in accordance with previous European reports, which have identified ST398 as the main clone colonizing horses admitted to veterinary hospitals [2]. This clone has also been reported in Portugal, in pigs, as a nasal colonizer and as the causative agent of an exudative epidermitis outbreak [12,15].

4. Discussion

In this study, we report the first isolation of MRSA strains from nasal swabs of purebred Lusitano horses. Although not very high (3%), the frequency of colonization found here was within the prevalence rate found by other authors, which ranged from 0% to 10.9% [2]. No association between MRSA carriage and healthy/sick horses could be made. One could expect that horses with RAO would have

a higher rate of nasal MRSA colonization, as there is a respiratory airway inflammation and horses tend to have flared nostrils. Yet, if we look in the human medicine literature, asthma (a disease with a similar pathophysiology to RAO) has only been recognized as a risk factor for methicillin-susceptible *S aureus* carriage and not for MRSA [16]. This could indicate that the type of pathology presented by the animals is not related to the MRSA carriage. Nevertheless, further studies are necessary to confirm this.

Considering the risk factors known for MRSA colonization in horses [1,2], only one was recognized: both MRSA-positive animals lived on a farm that housed more than 20 equines. Furthermore, the MRSA-colonized horses were from different regions: one was from Alter do Chão (Alentejo region) and the other one from Santo Estevão (Ribatejo region). The two horses had no previous history of antimicrobial therapy within the past 30 days; they had no record of previous admission to a neonatal intensive care unit or other hospital unit other than the surgical service. There was no hospitalization of other horses from the affected farm in Alter do Chão. One other horse from Santo Estevão was simultaneously hospitalized with the MRSA-positive horse, but was negative. However, previous colonization of the horses or the presence of colonized horses on both farms was unknown.

Isolate FMVA3/10 was clonally related to two human epidemic clones, the Pediatric clone and the New York/Japan clone. Pediatric MRSA clone (ST5-IV/VI) was first described in Portugal in 1992 as the dominant strain in a pediatric hospital in Lisbon [3]. Still in 2006, in a national surveillance study, this clone was isolated only once from a single patient in a hospital in the South of Portugal [17]. However, surprisingly, 4 years later in a study carried out in the Azores archipelago, the pediatric clone appeared as the second major clone isolated [3]. In contrast, the New York/Japan clone (ST5-II) was detected for the first time in a Portuguese hospital in 2005 as single isolate, but in 2006 was already accounting for 17% of the MRSA isolates and was the major clone in four hospitals in the Lisbon area [17]. This could induce the assumption that isolate FMVA3/10 could belong to the New York/Japan clone. Yet, the contrary seems more likely. Isolate FMVA3/10 harbors an SCC*mec* (type VI) that has only been described in the pedriatic clone and has a *spa* type (t062), which has only been associated, in Portugal, with ST5-VI [3]. Comparatively, all genome analysis by pulsed-field gel electrophoresis could give us some information, but only whole genome sequencing could enable us to assess the certain phylogeny, as it has been used to show that the ST5 clonal group emerged through a number of independent SCC*mec* transfer events in multiple geographic locations [18]. Either way, this isolate represents a human epidemic clone not previously reported among horses in Europe. This once again reinforces the fact that transmission of MRSA clones between horses and humans occurs and that there is a risk of horses acting as reservoirs of important resistant bacteria.

This is the first report of the detection of MRSA ST398 among horses in Portugal. It is in agreement with previous reports from Austria, Belgium, Canada, France, and the United Kingdom, indicating that ST398 is widespread among horses [1,2]. Isolate FMVA16/10 shares the same *spa* type (t011) as some of the previously described MRSA ST398 isolates from pigs and humans in Portugal [12,15]. Yet, this isolate does not share the same SCC*mec* (type IV) as the previous strains described (type V), which is in accordance with the theory that MRSA ST398 from horses are unrelated to the other isolates and probably have evolved independently by acquisition of a different SCC*mec* element [19].

The antimicrobial resistance patterns detected in the equine isolates (Table 2) match the current proposed antimicrobials in the guidelines for the treatment of infections in horses [20]. Gentamicin, tetracycline (in the form of oxytetracycline), erythromycin, and trimethoprim (in the form of trimethoprim in combination with a sulphonamide) are first choice antimicrobials in the absence of an antimicrobial susceptibility result or a pending culture [20]. Clindamycin is the only systemic antimicrobial, which is not used in equine medicine, but to which resistance is easily explainable because the *erm* genes confer resistance not only to macrolides but also to lincosamides. Fusidic acid, a topical antimicrobial, is often used in equines for the treatment of skin infections. To the best of our knowledge, this is the first report of *fusC*-mediated resistance in *S aureus* of equine origin.

5. Conclusion

This is the first report on the detection of MRSA of equine origin in Portugal. Horses can act as a reservoir of both human- and animal-adapted epidemic MRSA clones and thus influence animal-to-animal and animal-to-human MRSA dissemination. Furthermore, horses can be reservoirs of resistance genes that can be transferred to other important bacteria. Finally, equine MRSA carriage is by itself a hazard for the development of a multidrug-resistant animal infection.

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Each activity involving horses was regulated by the Ethical Commission of the Faculty of Veterinary Medicine of the Technical University of Lisbon according to Law (D.L. 129/92).

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3.1.2 First description of *fexA*-positive meticillin-resistant *Staphylococcus aureus* ST398 from calves in Portugal

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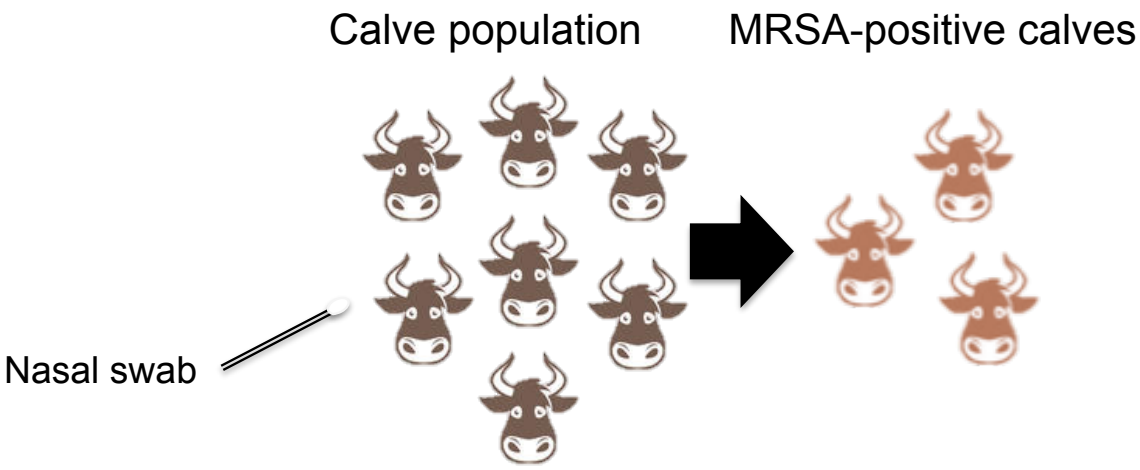

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Letter to the Editor

First description of *fexA*-positive meticillin-resistant *Staphylococcus aureus* ST398 from calves in Portugal

Sir,

Meticillin-resistant *Staphylococcus aureus* (MRSA) primarily causes human diseases, and food-producing animals are known to be reservoirs of MRSA clonal complex (CC) 398 [1,2]. MRSA CC398 has become a rapidly emerging cause of human infections, most often associated with livestock exposure [2]. A direct association between animal and human MRSA carriage has been established [3]. Until now, no data were available regarding MRSA in calves in Portugal.

In this study, nasal swabs were taken from randomly selected breeding calves at two closed-cycle farms from distinct regions of Portugal. In total, 247 nasal swabs from Farm A and 60 from Farm B were cultured as previously described [4]. Antimicrobial susceptibility testing was performed using the broth microdilution method [MicroScan® PM21; Siemens, Erlangen, Germany; and VetMIC® (Large Animals); National Veterinary Institute, Uppsala, Sweden] for the following antibiotics: chloramphenicol; ciprofloxacin; erythromycin; florfenicol; fosfomycin; fusidic acid; gatifloxacin; gentamicin; levofloxacin; linezolid; moxifloxacin; mupirocin; netilmicin; oxacillin; penicillin; quinupristin/dalfopristin; rifampicin; tetracycline; teicoplanin; trimethoprim/sulfamethoxazole; and vancomycin. Results of susceptibility testing were interpreted according to Clinical and Laboratory Standards Institute (CLSI) standards. The *mecA* and/or *mecC* genes were identified by PCR (http://www.crl-ar.eu), and MRSA isolates were subjected to staphylococcal protein A (*spa*) typing (http://www.seqnet.org/), staphylococcal cassette chromosome *mec* (SCC*mec*) typing [5] and pulsed-field gel electrophoresis (PFGE) using *Apa*I restriction [4]. The results of PFGE were interpreted using BioNumerics software v.4.6 (Applied Maths, Sint-Martens-Latem, Belgium). The presence of *fexA*, *tet*(K), *tet*(M), *erm*(A), *erm*(B) and *erm*(C) genes was studied by PCR. One strain (VF30T1) was randomly chosen for characterisation using an *S. aureus* Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany).

Six MRSA isolates from Farm A were identified as CC398, *spa* type t108 and SCC*mec* V (Table 1). No MRSA isolates were detected in Farm B. All isolates were indistinguishable by PFGE. None of the isolates carried the novel *mecC* gene. Susceptibility testing and PCR revealed resistance to tetracycline attributed to *tet*(K) and *tet*(M) genes, to chloramphenicol and florfenicol due to the presence of the *fexA* gene, and to fluoroquinolones in all MRSA isolates. One isolate (VF20T1) was also resistant to erythromycin and clindamycin and carried the *erm*(C) gene. Microarray-based genotyping analysis revealed that the isolate was positive for α and δ haemolysin, accessory gene regulator (*agr*) group 1 and capsule type 5 and carried a set of MSCRAMM (microbial surface components recognising adhesive matrix molecules) genes, including *clfA* and *clfB* (clumping factors A and B), *fnbA* and *fnbB* (fibronectin-binding proteins A and B), *fib* (fibrinogen-binding protein), *cna* (collagen-binding protein), *vwb* (von Willebrand factor-binding protein) and *ebpS* (elastin-binding protein). The isolate was negative for the Panton–Valentine leukocidin genes *lukF-PV* and *lukS-PV*, the immune evasion cluster (IEC), the toxic shock syndrome toxin 1 gene *tst*, exfoliative toxin genes as well as staphylococcal enterotoxin genes.

MRSA CC398 has been described in veal calves, cattle and bovine mastitis in Belgium, France, Germany, The Netherlands, Switzerland and the UK [3,5]. This is the first report of MRSA CC398 among calves in Portugal. These isolates carried the *fexA* gene, which has been previously described on a non-conjugative transposon (Tn*558*) in MRSA CC398 isolated from pigs and cattle in Germany [5]. The location of this phenicol exporter gene was not investigated in the current study. Resistance to fluoroquinolones in MRSA CC398, however, has only been reported sporadically [2], yet the isolates in this study were resistant to this class of

Table 1
Molecular characteristics and antimicrobial resistance profiles of meticillin-resistant *Staphylococcus aureus* (MRSA) isolated in six calves in Farm A (Portugal).

MRSA strain	Origin	Antimicrobial-associated co-resistance patterns[a]	Antimicrobial-associated co-resistance genes	SCC*mec* type	*spa* type	CC	PFGE cluster
VF97T0	Calf	CHL^r, FFC^r, FQ^r, TET^r	*fexA*, *tet*(M), *tet*(K)	V	t108	398	A
VF2T1	Calf	CHL^r, FFC^r, FQ^r, TET^r	*fexA*, *tet*(M), *tet*(K)	V	t108	398	A
VF10T1	Calf	CHL^r, FFC^r, FQ^r, TET^r	*fexA*, *tet*(M), *tet*(K)	V	t108	398	A
VF20T1	Calf	CHL^r, CLI^r, ERY^r, FFC^r, FQ^r, TET^r	*erm*(C), *fexA*, *tet*(M), *tet*(K)	V	t108	398	A
VF30T1	Calf	CHL^r, FFC^r, FQ^r, TET^r	*fexA*, *tet*(M), *tet*(K)	V	t108	398	A
VF42T1	Calf	CHL^r, FFC^r, FQ^r, TET^r	*fexA*, *tet*(M), *tet*(K)	V	t108	398	A

SCC*mec*, staphylococcal cassette chromosome *mec*; *spa*, staphylococcal protein A; CC, clonal complex; PFGE, pulsed-field gel electrophoresis; CHL, chloramphenicol; FFC, florfenicol; FQ, fluoroquinolones; TET, tetracycline; CLI, clindamycin; ERY, erythromycin; ^{r}, resistant.

[a] The following antimicrobials were tested: chloramphenicol; ciprofloxacin; erythromycin; florfenicol; fosfomycin; fusidic acid; gatifloxacin; gentamicin; levofloxacin; linezolid; moxifloxacin; mupirocin; netilmicin; oxacillin; penicillin; quinupristin/dalfopristin; rifampicin; tetracycline; teicoplanin; trimethoprim/sulfamethoxazole; and vancomycin.

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antimicrobials. Interestingly, Farm A used enrofloxacin routinely (three times 5-day course of 10 mg/kg enrofloxacin separated by 5 days without antimicrobial) to avoid respiratory and/or gastrointestinal infections. Farm B, on the other hand, administered gamithromycin for the same purpose. Fluoroquinolone administration is known to be a risk factor for MRSA infection in companion animals [1], so the use of enrofloxacin in Farm A could have favoured the spread of this MRSA clone. No information was available regarding usage of other antimicrobial agents that could have selected (β-lactams) or co-selected (e.g. florfenicol) for the occurrence of this multidrug-resistant clone.

Although these isolates did not carry the IEC known to be important for human colonisation, these strains still harboured a high number of virulence factors, which could still enable these MRSA to colonise and subsequently infect the human host. This fact and the multidrug resistance pattern identified in this study enhance the importance of livestock animals as a reservoir of antimicrobial resistance.

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Conflict of interest

No conflict of interest to declare.

Ethical approval

Not required.

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3.1.3 Clonal diversity, antimicrobial and biocide susceptibility patterns among human, animal and environmental methicillin-resistant *Staphylococcus aureus* in Portugal

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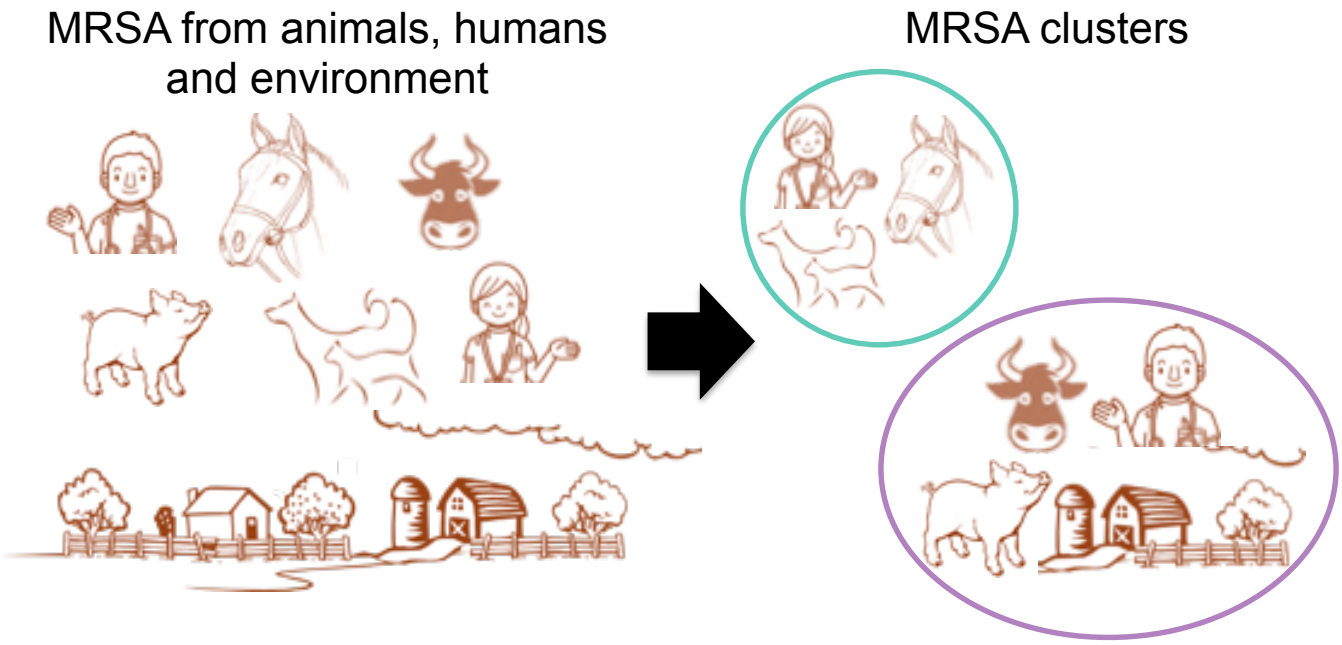

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Clonal diversity, virulence patterns and antimicrobial and biocide susceptibility among human, animal and environmental MRSA in Portugal

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Objectives: The objective of this study was to identify the *Staphylococcus aureus* clonal types currently circulating in animals, humans in contact with animals and the environment in Portugal based on genetic relatedness, virulence potential and antimicrobial/biocide susceptibility.

Methods: Seventy-four *S. aureus* isolates from pets, livestock, the environment and humans in contact with animals were characterized by SCC*mec* typing, *spa* typing, PFGE and CC398-specific PCR, by antimicrobial and biocide susceptibility testing and by detection of resistance genes and genes for efflux pumps. Representative strains were analysed by DNA microarray and MLST.

Results: The *S. aureus* isolates represented 13 *spa* types and 3 SCC*mec* types and belonged to three clonal complexes (CC5, CC22 and CC398). Most of the isolates were multiresistant and harboured the resistance genes that explained the resistance phenotype. The *qacG* and *qacJ* genes for biocide resistance were detected in 14 isolates (all MRSA CC398), while 4 isolates (3 CC5 and 1 CC22) had insertions in the −10 motif of the *norA* promoter. Isolates of the clonal lineages associated with pets (CC5 and CC22) harboured specific sets of virulence genes and often a lower number of resistance genes than isolates of the clonal lineage associated with livestock animals (CC398).

Conclusions: We found, for the first time in animals in Portugal, four strains belonging to CC5, including ST105-II, a lineage that has been previously reported as vancomycin-resistant *S. aureus* in Portugal. Moreover, for the first time the *qacG* and *qacJ* genes were detected in MRSA CC398 strains. Active surveillance programmes detecting MRSA not only in livestock animals but also in companion animals are urgently needed.

Keywords: *mecA*, staphylococci, public health, CC5, CC398, CC22

Introduction

Staphylococcus aureus, especially MRSA, are a major problem in the healthcare system and are also disseminated into the community.[1] In Portugal, a country with a high prevalence of nosocomial MRSA, MRSA of the clonal complexes CC22 and CC5 are the main clones causing infections in people attending healthcare centres and EMRSA-15 (ST22-IVh) accounts for more than 50% of the total isolates in hospitals.[1] The first European vancomycin-resistant *S. aureus* (VRSA), a CC5 MRSA clone ST105-II, was recently described in Portugal.[2] Animals can also become colonized and infected by MRSA, and might act as a reservoir for human infections.[3] In Portugal, colonization and infection with MRSA has been described in pigs, horses, calves, dogs and cats.[4–7] However, little is known about the potential of these strains to colonize/infect humans, especially those in contact with animals. Furthermore, animal MRSA strains can harbour antimicrobial resistance genes and/or efflux pumps that could potentially be transmitted to human MRSA strains, limiting the efficacy of antimicrobial/biocide treatment.[3,8,9]

The objective of this study was to identify and characterize the MRSA clonal types currently circulating in animals, humans in contact with animals and the environment in Portugal.

Materials and methods

Bacterial isolates

This study included all of the MRSA isolated at the Antibiotic Resistance Laboratory (Faculty of Veterinary Medicine, University of Lisbon) from 2001 to 2014, from all over Portugal (from routine diagnostic and national monitoring and surveillance programmes).[4–7] Infection (i) and colonization (c) isolates were obtained from pigs in 2008 (n=17, 11 i + 6 c), environmental dust samples from breeding pig sheds in 2008 (n=14), humans

Table 1. Characteristics of the 74 MRSA isolates

Clonal complex	Origin	SCC*mec* type	*spa* type	MLST	Resistance phenotype	Resistance genotype
CC5	dog (i)	II	t002	ST105	ERY, FQ, CLI, EtBr	*erm*(A), CAAT insertion at −10 motif of *norA* promotor
		II	t002*	ND	ERY, FQ, CLI, KAN, EtBr	*erm*(A), *erm*(B), *aadD*, CAAT insertion at −10 motif of *norA* promotor
	cat (i)	NT	t311	ST5	FUS	*fusC*
	horse (c)	NT	t062*	ST5	ERY, CLI, FUS	*erm*(C), *fusC*
	human (c)	IV	t002*	ND	ERY, KAN	*msr*(A), *aphA3*, *sat*
	human (c)	II	t002*	ND	ERY, CLI, FQ, KAN, SXT, EtBr	*erm*(A), *aadD*, CAAT insertion at −10 motif of *norA* promotor
CC22	dog (i)	IV	t032* (*n*=2)	ST22	FQ	
		IV	t025	ST22	ERY, CLI, FQ, EtBr	*erm*(C), GTTGTAATACAAT insertion at −10 motif of *norA* promotor
		IV	t2357 (*n*=2)	ST22, ND	ERY, CLI, FQ	*erm*(C)
		IV	t1865	ND	FQ	
	dog (c)	IV	t032	ND	FQ	
	cat (i)	IV	t032 (*n*=3)	ST22	FQ	
		IV	t032	ST22	ERY, CLI, FQ	*erm*(C), *mph*(C), *msr*(A)
	cat (c)	IV	t032 (*n*=2)	ND	FQ	
	human (c)	IV	t032* (*n*=4)	ND	FQ	
		IV	t1865 (*n*=2)	ND	FQ	
		IV	t020	ND	FQ	
		IV	t910	ND	FQ	
CC398	pig (i)	V	t011 (*n*=4)	ND	TET, CLI, TIA, SXT, TMP	*tet*(M), *tet*(K), *vga*(A), *dfrK*
		V	t011 (*n*=6)	ND	TET, CLI, TIA, SXT, TMP, EtBr	*tet*(M), *tet*(K), *vga*(A), *dfrK*, *qacG*
		V	t4571*	ND	TET, CLI, TIA, SXT, TMP, EtBr	*tet*(M), *tet*(K), *vga*(A), *dfrK*, *qacG*
	pig (c)	V	t011 (*n*=4)	ND	TET, CLI, TIA	*tet*(M), *tet*(K), *vga*(A)
		V	t011*	ND	TET, ERY, CLI, TIA, SXT, TMP, EtBr	*tet*(M), *tet*(K), *erm*(C), *vga*(A), *dfrK*, *qacG*
		V	t011	ND	TET, CLI, TIA, TMP, EtBr	*tet*(M), *tet*(K), *vga*(A), *dfrK*, *qacG*
	pig shed	V	t011	ND	TET	*tet*(M)
		V	t011 (*n*=2)	ND	TET, CLI, TIA, SXT, TMP	*tet*(M), *tet*(K), *vga*(A), *dfrK*
		V	t011	ND	TET, KAN, GEN, SXT, TMP	*tet*(M), *tet*(K), *aadD*, *aacA-aphD*, *dfrK*
		V	t108 (*n*=2)	ND	TET, CLI, TIA	*tet*(M), *vga*(A)
		V	t108	ND	TET, CLI, TIA	*tet*(M), *tet*(K), *vga*(A)
		V	t108	ND	TET, CLI, TIA, SXT, TMP	*tet*(M), *vga*(A), *dfrK*
		V	t108	ND	TET, ERY, CLI, TIA, SXT, TMP	*tet*(M), *tet*(K), *erm*(C), *vga*(A), *dfrK*
		V	t108 (*n*=2)	ND	TET, SXT, TMP	*tet*(M), *tet*(L), *dfrK*
		V	t108	ND	TET, SXT, TMP, EtBr	*tet*(M), *tet*(L), *dfrK*, *qacJ*
		V	t108	ND	TET, CHL, FFC, FQ	*tet*(M), *fexA*
		V	t1255*	ND	TET, CLI, TIA, APR	*tet*(M), *vga*(C), *apmA*
	calf (c)	V	t108 (*n*=4)	ND	TET, CHL, FFC, FQ	*tet*(M), *tet*(K), *fexA*
		V	t108*	ND	TET, KAN, CHL, FFC, FQ	*tet*(M), *tet*(K), *aphA3*, *fexA*
		V	t108	ND	TET, ERY, CLI, CHL, FFC, FQ	*tet*(M), *tet*(K), *erm*(C), *fexA*
	dog (i)	V	t108	ST398	TET, CHL, FFC, FQ	*tet*(M), *fexA*
	horse (c)	IV	t011	ND	TET, KAN, GEN, SXT, TMP	*tet*(M), *aacA-aphD*, *dfrK*
	human (c)	V	t011 (*n*=2)	ND	TET, ERY, CLI	*tet*(M), *tet*(K), *erm*(C)

V	t011	ND	TET, CLI, TIA	*tet*(M), *tet*(K), *vga*(A)	
V	t011	ND	TET, TMP	*tet*(M), *tet*(K), *dfrK*	
V	t011*	ND	TET, ERY, CLI, TIA, SXT, TMP, EtBr	*tet*(M), *tet*(K), *erm*(C), *vga*(A), *dfrK*, *qacG*	
V	t011 ($n=2$)	ND	TET, ERY, CLI, TIA, TMP, EtBr	*tet*(M), *tet*(K), *erm*(C), *vga*(A), *dfrK*, *qacG*	
V	t1255	ND	TET, TMP, EtBr	*tet*(M), *tet*(K), *dfrK*, *qacG*	

APR, apramycin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; FFC, florfenicol; FQ, fluoroquinolones; FUS, fusidic acid; GEN, gentamicin; KAN, kanamycin; ND, not determined; NT, non-typeable; SXT, sulfamethoxazole/trimethoprim; TET, tetracycline; TIA, tiamulin; TMP, trimethoprim.
Isolates with an asterisk were selected for microarray analysis.

in contact with animals in 2008–12 ($n=18$ c), calves in 2010 ($n=6$ c), dogs in 2008–14 ($n=10$, 1 i + 9 c), cats in 2001–14 ($n=7$, 5 i + 2 c) and horses in 2010 ($n=2$ c).

Antimicrobial susceptibility testing and resistance genes

The 74 isolates were routinely tested by broth microdilution for their antimicrobial susceptibility to a panel of antimicrobials (ampicillin, amoxicillin/clavulanic acid, chloramphenicol, ciprofloxacin or enrofloxacin, erythromycin, florfenicol, fusidic acid, gentamicin, kanamycin, penicillin, trimethoprim, trimethoprim/sulfamethoxazole and tetracycline). Genes encoding resistance to β-lactams, aminoglycosides, macrolides, lincosamides, tetracyclines, fusidic acid, phenicols and trimethoprim were detected by PCR.[8,9]

Biocide susceptibility and efflux pump genes

Determination of ethidium bromide (EtBr) MICs was used as a simple screening procedure for identifying strains with an increased efflux phenotype.[9] MICs of chlorhexidine acetate, benzalkonium chloride and triclosan were determined to further characterize the efflux phenotype. *S. aureus* ATCC 29213 was used as a quality-control strain. The detection of the biocide efflux pump genes *norA* and its promoter region, *qacA/B*, *smr*, *qacG*, *qacH* and *qacJ* was performed by PCR and sequencing of strains with high EtBr MICs (>8 mg/L).[9]

Molecular typing

All strains were assigned a *spa* type through the *spa* server (http://www.ridom.de/spaserver/). The isolates were assigned to clonal complexes according to the database of the *spa* server. These strains were also subjected to ST398-specific PCR and SCC*mec* typing using primers described previously.[8] CC5 and CC22 strains were compared by SmaI PFGE, while CC398 strains were compared by ApaI PFGE, using a previously described protocol.[8] Nine strains were subjected to MLST. Eleven strains were randomly chosen for characterization using the *S. aureus* Genotyping Kit 2.0 (Alere Technologies GmbH, Jena, Germany).

Statistical analysis

The comparison of groups of categorical data was performed using the Fisher's exact test with a level of significance set at 0.05, using SPSS v20 (IBM, New York, USA).

Results

Overall, 13 *spa* types were identified (Table 1). According to the *spa* server, four of these *spa* types were associated with CC398 ($n=47$), six were linked to CC22 ($n=21$) and three were associated with CC5 ($n=6$). These assignments were confirmed by results from MLST and CC398-specific PCRs. The PFGE patterns of all isolates belonging to the same clonal complex showed >80% similarity (Figures S1 and S2, available as Supplementary data at *JAC* Online). The CC22 isolates had SCC*mec* type IV and the CC398 isolates type V ($n=46$) and type IV ($n=1$). Among the CC5 isolates, three had SCC*mec* type II, one had type IV and two carried non-typeable SCC*mec* elements, which are currently sequenced. All CC398 strains were resistant to tetracycline due to the presence of the *tet*(M) gene alone or in combination with *tet*(K) or *tet*(L) genes. All CC22 and bovine CC398 strains were fluoroquinolone resistant. One porcine, one canine and the six bovine CC398 were resistant to chloramphenicol and florfenicol due to the presence of the *fexA* gene. Genes *dfrK* and *vga*(A) were present in

Table 2. Virulence characteristics of the 11 *S. aureus* characterized by the *S. aureus* Genotyping Kit 2.0 (Alere)

Origin	Clone	*agr* group	Haemolysins and leukotoxins	Enterotoxins	IEC
Dog (i)	CC5-t002-II	II	*hla*, *hlb*, *hld*, *lukF*, *lukS*, *lukD*, *lukE*	*sed*, *seg*, *sei*, *sej*, *sem*, *sen*, *seo*, *seu*, *ser*, *egc*	*chp*, *sak*, *scn*
Human (c)	CC5-t002-II	II	*hla*, *hlb*, *hld*, *lukF*, *lukS*, *lukD*, *lukE*	*sed*, *seg*, *sei*, *sej*, *sem*, *sen*, *seo*, *seu*, *ser*, *egc*	*chp*, *sak*, *scn*
Human (c)	CC5-t002-IV	II	*hla*, *hlb*, *hld*, *lukF*, *lukS*, *lukD*, *lukE*	*sea*, *seg*, *sei*, *sej*, *sem*, *sen*, *seo*, *seu*, *ser*, *egc*	*sak*, *scn*
Horse (c)	CC5-t062-nt	II	*hla*, *hlb*, *hld*, *lukF*, *lukS*, *lukD*, *lukE*	*seg*, *sei*, *sem*, *sen*, *seo*, *seu*, *egc*	*chp*, *sak*, *scn*
Dog (i)	CC22-t032-IV	I	*hla*, *hlb*, *hld*, *lukF*, *lukS*	*sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seu*, *egc*	—
Human (c)	CC22-t032-IV	I	*hla*, *hlb*, *hld*, *lukF*, *lukS*	*sec*, *seg*, *sei*, *sel*, *sem*, *sen*, *seo*, *seu*, *egc*	*chp*, *sak*, *scn*
Calf (c)	CC398-t108-V	I	*hla*, *hld*, *lukF*, *lukS*	—	—
Environmental dust	CC398-t1255-V	I	*hla*, *hld*, *lukF*, *lukS*	—	—
Pig (i)	CC398-t4571-V	I	*hla*, *hld*, *lukF*, *lukS*	—	—
Human (c)	CC398-t011-V	I	*hla*, *hld*, *lukF*, *lukS*	—	—
Pig (c)	CC398-t011-V	I	*hla*, *hld*, *lukF*, *lukS*	—	—

almost all porcine and environmental MRSA CC398 strains. All strains were susceptible to vancomycin.

Eighteen strains had high MICs of EtBr: 13 (all CC398) carried a *qacG* gene and had MICs of 16 mg/L, 1 strain (CC398) carried a *qacJ* gene and had an MIC of 32 mg/L and in 4 strains (3 CC5 and 1 CC22) the *norA* gene had an insertion of sequence CAAT ($n=3$) or GTTGTAATACAAT ($n=1$) in the −10 motif of the *norA* promoter and had MICs of 32 mg/L. MICs of benzalkonium chloride ranged from ≤0.125 to 4 mg/L and MICs of chlorhexidine acetate ranged from ≤0.125 to 1 mg/L. All strains had low MICs of triclosan (≤0.125 mg/L).

The main virulence characteristics of the 11 *S. aureus* characterized by the *S. aureus* Genotyping Kit 2.0 are summarized in Table 2. MRSA CC5 strains belonged to *agr* type II and the others to *agr* type I. All strains carried the following virulence genes: *isaB*, *isdA*, *hsdSx*, *aur*, *sspA*, *sspB*, *sspP*, *sdrC*, *hysA1*, *setC*, *ssl02*, *cap5*, *icaA*, *icaC*, *icaD*, *vwb*, *bbp*, *cflA*, *cflB*, *ebpS*, *eno*, *fib*, *fnbA*, *map*, *splA* and *splB*. None of the strains carried the ACME locus, the epidermal cell differentiation inhibitor (*edinA*, *edinB* or *edinC*), exfoliative toxins (*etA*, *etB* or *etD*), biofilm-associated protein (*bap*) or toxic shock syndrome toxic 1 (*tsst-1*). The main differences in the carriage of virulence genes were detected in the enterotoxins, haemolysins, leukotoxins and immune evasion cluster (IEC) (Table 2). Interestingly, the human MRSA CC22-t032 strain carried the IEC, while the dog MRSA CC22-t032 strain did not.

Pet-associated MRSA (CC5 and CC22) were significantly more likely to carry enterotoxin genes [*seg* ($P=0.002$), *sei* ($P=0.002$), *sem* ($P=0.002$), *sen* ($P=0.002$), *seo* ($P=0.002$), *seu* ($P=0.002$) and enterotoxin gene cluster (*egc*; $P=0.002$)] and staphylokinase gene [*sak*; $P=0.015$)], while livestock-associated MRSA CC398 were significantly more likely to carry efflux pumps and particular antimicrobial resistance genes [*qacG* ($P=0.003$), *dfrK* ($P=0.0001$), *tet*(K) ($P=0.0001$), *tet*(M) ($P=0.0001$) and *vga*(A) ($P=0.0001$)].

Discussion

During recent years, we have observed a significant increase in the number of MRSA descriptions in animals in several countries, including Portugal,[4–7] despite only isolated studies describing MRSA in animals and a single surveillance study (European Union-wide baseline survey on MRSA conducted in 2008 in breeding pig holdings) conducted in Portugal. Studies on the role of animals, especially pets, in the transmission of MRSA into the community are still lacking. In Denmark MRSA CC398 constituted 31% of all new MRSA cases in 2013 and patients in contact with live pigs are screened for MRSA colonization when entering the hospital setting.[10,11] In contrast, livestock-associated MRSA constitutes a small percentage of the overall MRSA burden in Portugal and active screening does not include patients with animal contact.[12] This study showed that people in direct contact with animals (owners, handlers and veterinary personnel) carried similar MRSA clones as the animals they were in contact with. Especially worrying was the fact that humans in contact with companion animals carried clones (CC5 and CC22) circulating in hospitals and the community.[1] We found, for the first time in animals in Portugal, four strains belonging to CC5. One of these strains belonged to ST105-II, the same lineage as the recently described VRSA in Portugal.[2] VRSA isolates from other countries also belonged to CC5.[13] In addition to the possibility of pets being a reservoir and distributer of VRSA, companion animals can also carry *vanA*-carrying VRE and thereby raise the chances of acquisition of the *vanA* gene cluster.[14]

MRSA CC22 and CC5 strains carried significantly more enterotoxins than MRSA CC398, including *egc*, and at least one IEC gene. One dog MRSA CC22-t032 strain did not carry IEC genes, which might suggest host adaptation. Still a recent study found no significant difference in the presence or absence of the IEC between human and companion animal isolates when correcting for shared evolutionary history, suggesting that IEC conferred isolates with an extended host spectrum.[15] Companion animals seem to carry *S. aureus* clonal linages that are more virulent to humans than livestock animals, and so active surveillance of MRSA in companion animals seems to be urgently needed.

We found for the first time, to the best of our knowledge, the *qacG* and *qacJ* genes in MRSA CC398 strains. The *qacG* gene has been described in porcine MRSA isolates from clonal lineage ST9 in Hong Kong[16] and both genes have also been detected among staphylococci of bovine and caprine origin in Norway.[17] Biocides are extensively used in animal husbandry, including quaternary ammonium compounds.[14] The acquisition of *qacG* and *qacJ* by MRSA CC398, usually carried on plasmids, may aid to the persistence of MRSA in the environment, making the eradication of MRSA CC398 more difficult.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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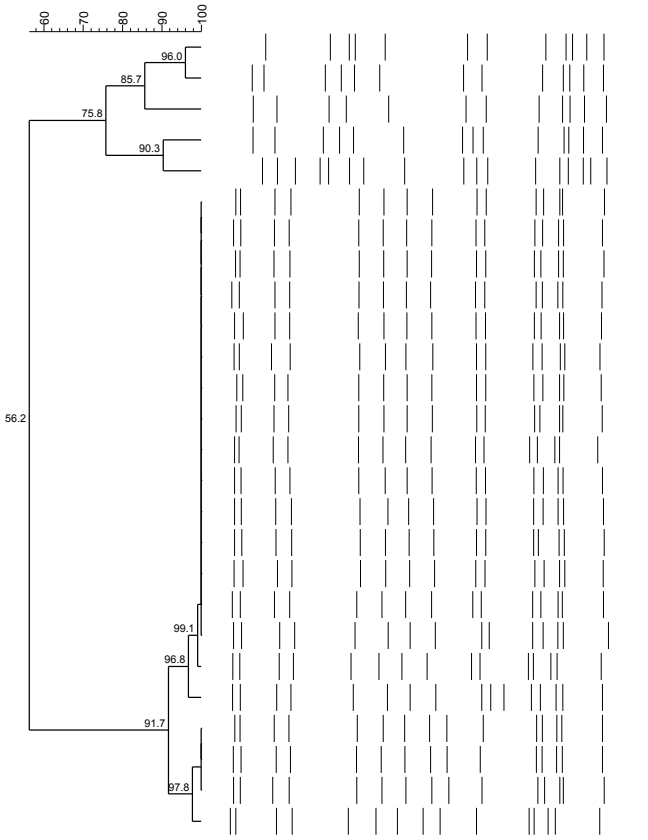
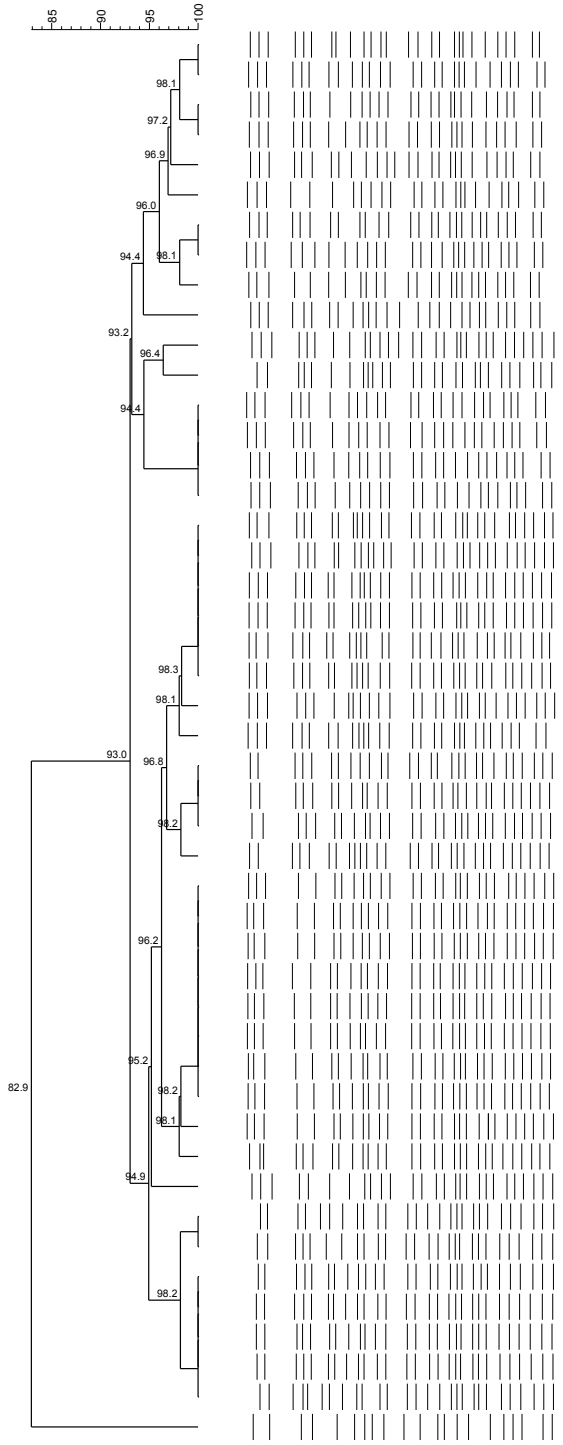
Supplementary Figure 1. Dendogram of chromosomal DNA digested with *Sma*I of MRSA CC5 and CC22 strains. Pulsed-field cluster determination using a Dice similarity coefficient with an optimization of 1% and a band tolerance setting of 1.7%.



Source	*spa* type	CC	SCC*mec*
Human	t002	CC5	II
Dog	t002	CC5	II
Dog	t002	ST105	II
Horse	t062	ST5	new
Cat	t311	ST5	new
Human	t032	CC22	IV
Cat	t032	CC22	IV
Cat	t032	CC22	IV
Dog	t032	CC22	IV
Human	t032	CC22	IV
Human	t032	CC22	IV
Human	t1865	CC22	IV
Dog	t2357	CC22	IV
Human	t020	CC22	IV
Human	t032	CC22	IV
Human	t910	CC22	IV
Human	t1865	CC22	IV
Dog	t1865	CC22	IV
Cat	t032	ST22	IV
Cat	t032	ST22	IV
Dog	t2357	ST22	IV
Dog	t025	ST22	IV
Dog	t032	ST22	IV
Cat	t032	ST22	IV
Cat	t032	ST22	IV
Dog	t032	ST22	IV

Figure 2. Dendogram of chromosomal DNA digested with *Apa*I of MRSA CC398 strains. Pulsed-field cluster determination using a Dice similarity coefficient with an optimization of 1% and a band tolerance setting of 1.7%.



Source	*spa* type	CC	Sample mode	SCC*mec*
Environmental dust	t108	CC398	Dust sample	V
Environmental dust	t108	CC398	Dust sample	V
Environmental dust	t1255	CC398	Dust sample	V
Environmental dust	t108	CC398	Dust sample	V
Environmental dust	t011	CC398	Dust sample	V
Environmental dust	t108	CC398	Dust sample	V
Environmental dust	t011	CC398	Dust sample	V
Environmental dust	t011	CC398	Dust sample	V
Environmental dust	t108	CC398	Dust sample	V
Environmental dust	t011	CC398	Dust sample	V
Environmental dust	t108	CC398	Dust sample	V
Environmental dust	t108	CC398	Dust sample	V
Swine	t011	CC398	Nasal swab	V
Swine	t011	CC398	Nasal swab	V
Swine	t011	CC398	Nasal swab	V
Swine	t011	CC398	Nasal swab	V
Bovine	t108	CC398	Nasal swab	V
Bovine	t108	CC398	Nasal swab	V
Bovine	t108	CC398	Nasal swab	V
Bovine	t108	CC398	Nasal swab	V
Bovine	t108	CC398	Nasal swab	V
Bovine	t108	CC398	Nasal swab	V
Human	t011	CC398	Nasal swab	V
Environmental dust	t108	CC398	Dust sample	V
Human	t011	CC398	Nasal swab	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t4571	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Swine	t011	CC398	Exsudative epidermitis	V
Environmental dust	t108	CC398	Dust sample	V
Human	t011	CC398	Nasal swab	V
Dog	t108	ST398	Skin	V
Human	t011	CC398	Nasal swab	V
Human	t1255	CC398	Nasal swab	V
Human	t011	CC398	Nasal swab	V
Human	t011	CC398	Nasal swab	V
Human	t011	CC398	Nasal swab	V
Equine	t011	CC398	Nasal swab	IV

3.2 Part 2 – Epidemiology, antimicrobial and biocide susceptibility of staphylococci isolated from animals in Portugal

3.2.1 Biocide and antimicrobial susceptibility of methicillin-resistant staphylococcal isolates from horses

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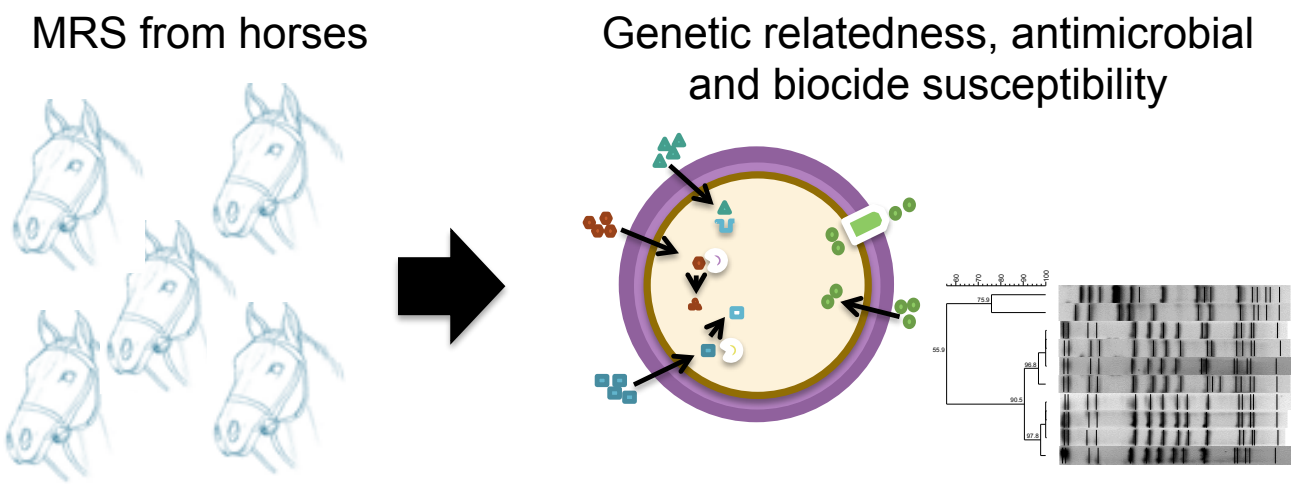

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Short communication

Biocide and antimicrobial susceptibility of methicillin-resistant staphylococcal isolates from horses

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ABSTRACT

The aim of this study was to evaluate the biocide and antimicrobial susceptibility of methicillin-resistant staphylococcal isolates from horses. Fourteen methicillin-resistant staphylococci (MRS) were subjected to an extensive genotype characterization, including SCC*mec*, *dru*, *spa*, PFGE and MLST typing. Antimicrobial susceptibility testing was performed and resistance genes were detected by PCR. Minimum bactericidal concentrations (MBCs) of four biocides [chlorhexidine acetate (CHA), benzalkonium chloride (BAC), triclosan (TCL) and glutaraldehyde (GLA)] were determined following the recommendations of document NF EN 1040. The presence of *qac* and *sh-fabI* genes was investigated by PCR. Several antimicrobial resistance patterns and genes were detected. When MRS strains were exposed for a longer period of time, a lower concentration of the biocide was needed to achieve lethality. TCL had the lowest MBC values. All MBC values were lower than the recommended in-use concentrations for veterinary medicine. *S. haemolyticus* and *S. cohnii* subsp. *cohnii* carried plasmid-borne *qacA* and *sh-fabI* or *qacB* and a *qacH*-like genes, respectively. Biocides appear to be a reliable antiseptic option against MRS, since even in the presence of bacterial efflux mechanisms, the recommended concentration is much higher than the *in vitro* MBC.

1. Introduction

Methicillin-resistant staphylococci (MRS) have been frequently isolated from the anterior nares of healthy animals, including horses (Moodley and Guardabassi, 2009; Weese and van Duijkeren, 2010). These bacteria can be transmitted from animals to humans, especially to veterinarians and animal handlers which are in close contact (Moodley and Guardabassi, 2009). Although staphylococci are a diverse group of commensals inhabiting the skin and mucous membranes of humans and animals, some species, like *Staphylococcus aureus*, are known as important human pathogens (Weese and van Duijkeren, 2010). In horses, *S. aureus* and coagulase-negative staphylococci (CoNS) are responsible for skin/soft tissue, joint and incision infections (Moodley and Guardabassi, 2009; Weese and van Duijkeren, 2010). These infections are usually treated with systemic and/or topical antibiotics and/or topical antiseptic preparations (Bjorland et al., 2003; Schnellmann et al., 2006). In contrast to antibiotics, most of the biocide preparations used as antiseptics and disinfectants, are commercially available

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without prescription by a veterinarian (Bjorland et al., 2003). This has raised concerns about the possible development of "biocide resistance" and cross-resistance to antibiotics (e.g. via mechanisms acting on both biocides and antibiotics) (SCENIHR, 2010; Ciusa et al., 2012). Different mechanisms to evade the toxic activity of biocides have been recognized (Cerf et al., 2010). One of those mechanisms, which can be encoded on the bacterial chromosome or on plasmids, is overexpression of efflux pumps (SCENIHR, 2010). In *Staphylococcus* species several efflux pumps have been recognized, but QAC efflux proteins seem to be the most widespread (Bjorland et al., 2003; Couto et al., 2008; Vali et al., 2008). A recent report identified a novel transferable mechanism of reduced biocide susceptibility to triclosan, mediated by the *sh-fabI* gene (Ciusa et al., 2012). The Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) has recommended the determination of minimum bactericidal concentrations (MBCs) as the appropriate methodology that allows the comparison of lethality in susceptible and resistant strains (SCENIHR, 2010). This determination must involve the use of a neutralizing agent or the removal of the biocide, otherwise it will provide an over-estimation of the lethality of the compound (SCENIHR, 2010; Cerf et al., 2010).

In the present study, we characterized the antimicrobial resistance and biocide susceptibility of a collection of MRS isolates from horses and studied the genetic relatedness of the isolates.

2. Materials and methods

2.1. Bacterial isolates

From March 2008 to October 2010, a study was performed in horses entering the Equine Unit of the Faculty's Veterinary Hospital. The objective was to determine the prevalence of MRSA nasal colonization in horses. Twenty horses were admitted for elective surgery – castration or as part of a control group or were admitted with a disease other than infection (30 samples from horses with recurrent airway obstruction (RAO), 18 from horses with different respiratory diseases and 13 from horses with equine gastrointestinal ulcer syndrome (EGUS)). None of the horses had been treated with an antibiotic or an antiseptic prior to sample collection. The presumptive colonies were identified as staphylococci by routine methods. All the *Staphylococcus* spp. strains growing on ChromID™ MRSA (bioMérieux, Marcy L'Etoile, France) were stored in brain–heart infusion broth with 20% sterile glycerol at −20 °C. For this study, the 14 MRS strains included were classified by BBL™ Crystal™ Gram-Positive ID Kit (Becton, Dickinson and Company, Maryland, USA). They comprised eight *Staphylococcus sciuri*, two *Staphylococcus aureus*, and single strains of *Staphylococcus lentus*, *Staphylococcus fleurettii*, *Staphylococcus haemolyticus* and *Staphylococcus cohnii* subsp. *cohnii*. Methicillin resistance was confirmed by amplification of the *mecA* gene (Schnellmann et al., 2006). Since isolates A4C/08, A7C/08 and A36B/08 could not be properly identified by this identification system, they were subjected to 16S rDNA gene sequencing.

2.2. Molecular typing

All MRSA strains were tested by PCR for the presence of *lukF*/*lukS* genes encoding Panton-Valentine leukocidin (PVL) (Feβler et al., 2010) and *spa* types were assigned through the Ridom web server (http://www.ridom.de/spaserver/). These strains were also subjected to ST398 specific PCR using primers described by van Wamel and colleagues in 2010. Multi-locus sequence typing (MLST) was performed in the non-ST398 MRSA strains. SCC*mec* and *dru* types were determined as described previously (Feβler et al., 2010). The *S. sciuri* strains were compared by SmaI PFGE, using a previously described protocol (Feβler et al., 2010).

2.3. Antimicrobial susceptibility testing and detection of resistance genes

Susceptibility to a range of antibiotics was determined by broth microdilution, following CLSI standards (CLSI, 2008, 2011). EUCAST guidelines (EUCAST, 2009) were used for fusidic acid and mupirocin minimal inhibitory concentration (MIC) interpretation. E-test strips (Liofilchem, Roseto degli Abruzzi, Italy) were used for fusidic acid MIC determination. β-Lactamase production was detected by using the lyophilized nitrocefin SR00112 (Oxoid, Hampshire, United Kingdom) broth method, using *S. aureus* ATCC 29213 and *S. aureus* ATCC 25923 as controls. Genes previously reported for resistance to β-lactams, aminoglycosides, macrolides, lincosamides, tetracyclines, pleuromutilins, fusidic acid, chloramphenicol and trimethoprim were detected by PCR (Schnellmann et al., 2006; Feβler et al., 2010).

2.4. Biocide susceptibility and detection of qac and sh-fabI genes

Ethidium bromide (EtBr) MIC determination was used as a simple screening procedure for identifying strains with an increased efflux phenotype (Couto et al., 2008). MIC of chlorhexidine acetate (CHA), benzalkonium chloride (BAC), and triclosan (TCL) were determined to further characterize the efflux phenotype. TCL was included because it is used for the control of MRSA carriage among human patients and so monitoring of susceptibility is very important. MICs of glutaraldehyde (GLA) were not determined since an increase in the MIC of GLA is not related to any known mechanism of reduced susceptibility. The determination of MBC of the four biocides (CHA, BAC, TCL and GLA) was performed according to Narui and colleagues (2007), and following the recommendations of document NF EN 1040 (AFNOR, 2006). In brief, each cell suspension was inoculated with the biocide with an exposure time of 5, 30 and 60 min at 20 °C. Then the bacteria-biocide mixture was transferred into the neutralization medium for 5 min and finally was inoculated into Muller–Hinton broth without biocide. Bacterial growth was evaluated after incubation at 37 °C for 24 h. *S. aureus* ATCC 29213 and *S. aureus* ATCC 6538 were used as quality controls. The detection of the biocide resistance genes *qacA/B*, *smr*, *qacG*, *qacH* and *qacJ* was performed by

Table 1
Molecular characteristics, antimicrobial resistance profiles, MICs of dyes (ethidium bromide) and biocides (benzalkonium chloride, chlorhexidine acetate and triclosan), and genes associated with reduced biocide susceptibility of methicillin-resistant staphylococci isolated from horses.

Isolate	Species	Origin	SCC*mec* type	*dru* type	Antimicrobial resistance profiles	Antimicrobial resistance genes	MIC (mg/L)				Genes associated with reduced susceptibility to biocides
							EtBr	BAC	CHA	TCL	
1	*S. cohnii* subsp. *cohnii*	Horse with RAO	III	nt	ERY, TIA	*msr*(A), *mph*(C)	128	2	1	2	*qacB*, *qacH-like*
2	*S. lentus*	Horse with RAO	nt	dt13v	CHL, TIA	cat_{pC221}	0.5	0.5	0.5	0.007	–
3	*S. fleurettii*	Horse with RAO	III	dt11a	TIA	–	0.5	0.5	0.5	0.007	–
4	*S. sciuri*	Horse with RAO	III	dt11a	TET, TIA	*tet*(K)	0.5	2	1	≤0.003	–
5	*S. sciuri*	Healthy horse	III	dt11a	TET, TIA	*tet*(K)	0.5	1	1	≤0.003	–
6	*S. sciuri*	Horse with RAO	III	dt11a	TET, TIA	*tet*(K)	0.5	2	1	≤0.003	–
7	*S. sciuri*	Healthy horse	III	dt11a	TET, TIA	*tet*(K)	0.5	2	1	≤0.003	–
8	*S. sciuri*	Horse with RAO	III	dt11a	TIA	–	0.5	2	1	≤0.003	–
9	*S. sciuri*		III	dt11a	TET, TIA	*tet*(K)	0.5	2	1	≤0.003	–
10	*S. sciuri*	Horse with EGUS	nt	nt	TIA	–	1	1	2	≤0.003	–
11	*S. sciuri*	Healthy horse	nt	dt11a	TET, TIA	*tet*(K)	0.5	0.5	0.5	≤0.003	–
12	*S. haemolyticus*	Healthy horse	nt	dt11ay	CLI, ERY, GEN, KAN, ENR	*blaZ*, *erm*(C), *aacA-aphD*, *aphA-3*	64	2	1	4	*qacA*, *sh-fabI*
13	*S. aureus*	Healthy horse	IV	dt10q	GEN, KAN, TET, TMP	*blaZ*, *aacA-aphD*, *tet*(M), *dfrK*	2	0.5	0.5	≤0.003	–
14	*S. aureus*	Horse with EGUS	VI	nt	CLI, ERY, FUS	*blaZ*, *erm*(C), *fusC*	8	0.5	0.5	≤0.003	
RN4220	*S. aureus*						8	1	1	0.125	–
RN4220pNCP1	*S. aureus*						128	2	1	0.125	*qacB*
RN4220pNCP2	*S. aureus*						64	2	1	0.125	*qacA*
RN4220pNCP3	*S. aureus*						8	1	1	4	*sh-fabI*

Abbreviations: BAC: benzalkonium chloride; CC: clonal complex; CHA: chlorhexidine acetate; CHL: chloramphenicol; CLI: clindamycin; EGUS: equine gastrointestinal ulcer syndrome; ENR: enrofloxacin; ERY: erythromycin; EtBr: ethidium bromide; FUS: fusidic acid; GEN: gentamicin; KAN: kanamycin; nt: non typeable; RAO: recurrent airway obstruction; TCL: triclosan; TET: tetracyclin; TIA: tiamulin; TMP: trimethoprim.

PCR (Bjorland et al., 2005; Couto et al., 2008) and sequencing. The presence of *sh-fabI* was investigated by PCR using strain *S. aureus* M0091 as a positive control (Ciusa et al., 2012).

2.5. Transformation experiments

In order to characterize the biocide susceptibility profiles, plasmids from strains 1 and 12 (Table 1) were transferred into *S. aureus* RN4220 by electrotransformation. Transformants were selected by incubation on brain–heart infusion agar plates, supplemented with 0.5 μg/mL TCL or 16 μg/mL EtBr. Transformants, which appeared after 24–48 h, were screened for their plasmid content and their antimicrobial susceptibility phenotype.

3. Results

Both MRSA isolates were PVL-negative and were classified as either ST5-t062-SCC*mec* VI or ST398-t011-SCC*mec* IV (Table 1). The SCC*mec* and *dru* types of all MRS are listed in Table 1. All *S. sciuri* isolates showed indistinguishable SmaI PFGE profiles, although the isolates included in this study were from individual horses, except for strains 8 and 9, which were isolated from the same horse. Isolates 8 and 9 differed, however, in their tetracycline resistance pheno- and genotype. This observation suggests that this clone of *S. sciuri* is widely disseminated among the horse population in Portugal or may be present in the veterinary hospital in which the horses have been sampled.

The antimicrobial resistance patterns and the resistance genes detected are summarized in Table 1. The three isolates carrying the *blaZ* gene, expressed the β-lactamase as detected by the nitrocefin test. None of the *vga* genes tested were identified in the MRCoNS, which exhibited high tiamulin MICs of 32 to >64 μg/mL.

The MIC values of three biocides (BAC, CHA and TCL) and one dye (EtBr) are summarized in Table 2. Two MRCoNS expressed an efflux phenotype and were *qacA/B*-positive (Table 1). Sequencing of the *qac*-amplicons identified a *qacA* gene in the *S. haemolyticus* isolate and a *qacB* and a *qacH*-like gene in the *S. cohnii* subsp. *cohnii* isolate. The *sh-fabI* gene was present only in the

Table 2
Minimum and maximum user concentration and contact time of biocides normally used in veterinary medicine.

Agent	User concentration (μg/mL) and contact time (min)[a]							
	Skin and wound		Environment		Surgical site		Hand scrubs	
Benzalkonium chloride	1000–2000	5–10	60–120	120	100–500	N.A.	500–1000	1–3
Glutaraldehyde	N.A.	N.A.	625–1250	120–240	N.A.	N.A.	N.A.	N.A.
Chlorhexidine acetate	150–40,000	5–10	N.A.	N.A.	40,000	5–10	1000–40,000	1–3

Abbreviations: min: minutes; N.A.: not applicable.
[a] This is based on commercial veterinary products available at the manufactures' websites and based on documents: European Medicines Agency. Committee for Veterinary Medicinal Products. Chlorhexidine. EMA, London, United Kingdom, 1996 (http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2009/11/WC500012062.pdf); European Medicines Agency. Committee for Veterinary Medicinal Products. Benzalkonium chloride. EMA, London, United Kingdom, 1997 (http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_-Limits_-_Report/2009/11/WC500010967.pdf).

aforementioned *S. haemolyticus* isolate. The MBC values for TCL were very low (≤0.125 μg/mL) and not within the concentration range tested (data not shown), except for isolates 1 and 12 (2–32 μg/mL). Nevertheless, in both cases the MBC values decreased over time, similar as was observed with the other three biocides. The *S. haemolyticus* strain had the highest MBC value to TCL at all times (32 μg/mL at 5 min, 16 μg/mL at 10 min and 8 μg/mL at 30 min) and carried the *sh-fabI* gene. Both *qacB*- and *qacA*-carrying plasmids (pNCP1 and pNCP2, respectively) were successfully transformed into *S. aureus* RN4220, using EtBr to select for the transformants. Transformation of *S. aureus* RN4220 with a plasmid from the *S. haemolyticus* strain, using TCL to select for transformants, confirmed that the *sh-fabI* gene was also located on a plasmid (pNCP3). None of the transformants was resistant to any of the antibiotics tested, suggesting that the biocide resistance genes were the only resistance genes on plasmids pNCP1, pNCP2 and pNCP3.

4. Discussion

In this study, we tested the biocides CHA, BAC, GLA and TCL against a collection of MRS isolates from horses. Two isolates (14%) showed an efflux phenotype (higher MIC values to EtBr compared to the wild-type *S. aureus* RN4220) and were *qacA/B* positive. QAC efflux pumps have been previously reported among staphylococci from horses and other farm animals (Bjorland et al., 2003, 2005). However, the occurrence of *qacA* or *qacB* genes among staphylococci of equine origin has not been described so far. In human medicine, the frequency of *qac* genes differs worldwide. Yet, studies performed in the United Kingdom found a frequency of *qacAB* genes of 10% to 20% among human MRSA strains (Vali et al., 2008), which is comparable to that found in this study. Similarly one study performed in MRS strains of bovine and caprine origin detected *qac* genes in 21% of the 127 dairy cattle herds and 10% of the 70 dairy goat herds that were screened (Bjorland et al., 2005). In the same study the *qac*-positive strains had MICs for BAC that ranged from 1 to 5 μg/mL (Bjorland et al., 2005), which is similar to our results.

Neither of the two *qacA/B* positive strains found in our study had high MBC values to BAC, CHA or GLA. Surprisingly the *qacA/B* positive strains had significantly higher MBC values to TCL than the *qac*-negative strains. The *qacB*-positive strain also harboured a *qacH*-like gene but no additive effect in the efflux phenotype was observed. No additive effect was also seen in a strain carrying a *qacA/B* plus a *smr* gene in a previous study (Bjorland et al., 2005). No cross-resistance to antibiotics was detected either, since neither of the two transformants harbouring the *qac* genes showed resistance to the antibiotics tested. As expected, when MRS strains were exposed for a longer period of time, a lower concentration of the biocide was needed to achieve lethality. TCL was the most effective biocide, since the lowest concentrations were needed to achieve killing. Yet in a previous study with the same methodology, TCL was the biocide with the highest MBCs against laboratory isolates of *S. aureus*, *Pseudomonas aeruginosa* and *Candida albicans* (Koburger et al., 2010). However, colonization isolates may have different susceptibilities than laboratory strains depending on the selective pressure they have been subjected to *in vivo*. As TCL is currently not approved for and consequently not used in equine medicine (Ciusa et al., 2012), the apparent lack of selective pressure may explain the high susceptibility of the equine MRS isolates. One MRS *S. haemolyticus* isolate harbored a plasmid-borne *sh-fabI* gene with a TCL MIC of 4 μg/mL and an MBC of 32 μg/mL (at 5 min). This observation was in accordance with a previous study, where the same MIC and MBC values were detected in a *S. aureus* strain carrying a plasmid-borne *sh-fabI* gene and in a *S. haemolyticus* strain harboring a chromosomal *sh-fabI* gene. This gene is responsible for a novel mechanism of diminished susceptibility to TCL with a high potential for horizontal gene transfer through plasmids (Ciusa et al., 2012). In our study the *sh-fabI*-gene was found in a plasmid and not in the chromosome of *S. haemolyticus*, as reported by Ciusa and colleagues (2012). The higher MIC and MBC values in the *S. cohnii* subsp. *cohnii* strain, and the absence of the *sh-fabI* gene may indicate that another mechanism is present, probably mutations in the original *fabI* gene, which have been previously described in *S. aureus* (Ciusa et al., 2012).

Overall, the MBC values of BAC, CHA and GLA were lower than the concentrations currently used in some commercial products (Table 2) or recommended for use in veterinary applications (Table 2). Nevertheless, when comparing the MBC values of the isolates of the present study with the concentration of BAC in human products (100 μg/mL) (Narui et al., 2007), five isolates with BAC MBCs of >128 μg/mL were detected. A few isolates with higher MBC results for CHA and BAC than the concentration used

in human products have already been reported (Narui et al., 2007). This is of notice, since transmission of MRS strains between horses, humans and their environments may occur.

Although antimicrobial therapy of MRS has become difficult due to the antimicrobial multidrug-resistance of many MRS isolates, biocides are still a bactericidal option not only for treatment of skin and wound infections, but also for decolonization purposes.

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3.2.2 Genetic Relatedness, Antimicrobial and Biocide Susceptibility Comparative Analysis of Methicillin-Resistant and -Susceptible *Staphylococcus pseudintermedius* from Portugal

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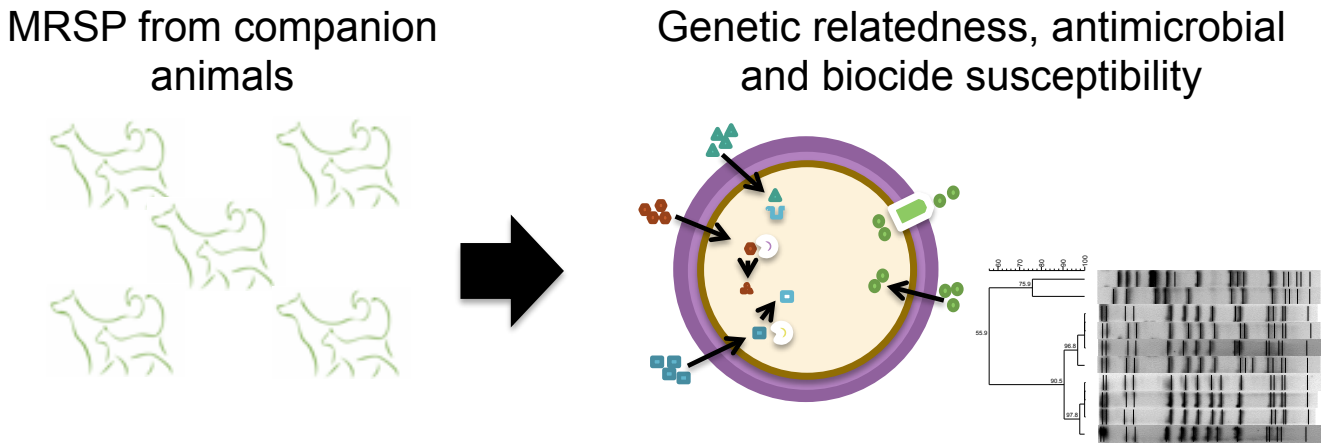

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Genetic Relatedness, Antimicrobial and Biocide Susceptibility Comparative Analysis of Methicillin-Resistant and -Susceptible *Staphylococcus pseudintermedius* from Portugal

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Forty methicillin-resistant and -susceptible *Staphylococcus pseudintermedius* (MRSP and MSSP, respectively) from colonization and infection in dogs and cats were characterized for clonality, antimicrobial, and biocide susceptibility. MSSP were genetically more diverse than MRSP by multi-locus sequence typing and pulsed-field gel electrophoresis. Three different *spa* types (t06, t02, t05) and two SCC*mec* types (II-III and V) were detected in the MRSP isolates. All MRSP and two MSSP strains were multidrug-resistant. Several antibiotic resistance genes (*mecA*, *blaZ*, *tet*(M), *tet*(K), *aac(6′)-Ie–aph(2′)-Ia*, *aph(3′)-III*, *ant(6)-Ia*, *sat4*, *erm*(B), *lnu*(A), *dfr*(G), and cat_{pC221}) were identified by microarray and double mutations in the *gyrA* and *grlA* genes and a single mutation in the *rpoB* gene were detected by sequence analysis. No differences were detected between MSSP and MRSP in the chlorhexidine acetate (CHA) minimum inhibitory concentrations (MICs). However, two MSSP had elevated MIC to triclosan (TCL) and one to benzalkonium chloride and ethidium bromide. One MSSP isolate harboured a *qacA* gene, while in another a *qacB* gene was detected. None of the isolates harboured the *sh-fabI* gene. Three of the biocide products studied had high bactericidal activity (Otodine®, Clorexyderm Spot Gel®, Dermocanis Piocure-M®), while Skingel® failed to achieve a five log reduction in the bacterial counting. *S. pseudintermedius* have become a serious therapeutic challenge in particular if methicillin- resistance and/or multidrug-resistance are involved. Biocides, like CHA and TCL, seem to be clinically effective and safe topical therapeutic options.

Introduction

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has emerged recently and has become a serious therapeutic challenge for veterinarians, due to multidrug resistance.[9,12,24,30] They are a major cause of skin and urinary tract, and hospital acquired infections in dogs and cats.[12,30] Originally, two major MRSP clones were found to spread in Europe (ST71-t02-SCC*mec* II-II) and North America (ST68-t06-SCC*mec* V).[4,24,30] Although more recent reports have yet identified other *S. pseudintermedius* lineages carrying the *mecA* gene,[11,22] methicillin-susceptible *S. pseudintermedius* (MSSP) tend to be genetically more diverse than MRSP.[2,4]

In addition to the *mecA* gene, MRSP isolates usually have mutations in the gyrase and topoisomerase genes, conferring resistance to fluoroquinolones[10] and several other genes, which mediate resistance to gentamicin, kanamycin, erythromycin, clindamycin, streptomycin, tetracycline and trimethoprim.[12,30] Resistance to rifampicin and chloramphenicol has also been reported in some MRSP strains.[12,17,30] This pattern of multidrug resistance is normally in contrast to what happens with MSSP.[12] Resistance to ampicillin and penicillin is often reported in MSSP isolates, but they are usually susceptible to the other antimicrobial classes.[12] Yet, treatment of MRSP infections is based on the same principles as MSSP infections, usually involving systemic and/or topic therapy. The difference lies on the number of antimicrobial options available for a successful treatment. While there are several antimicrobial options for MSSP therapy, some of the antimicrobials used for the treatment of MRSP infections are not licensed for veterinary use and considered "critically important" for human medicine by the World Health Organization.[32] Other antimicrobials, like rifampicin and

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chloramphenicol, are potentially toxic and have suboptimal pharmacological features for small animals.[12,17] For this reason, topical therapy, especially antiseptic preparations, has gained a renewed interest. Biocide topical therapy can be used as solo or as an adjuvant for the treatment of skin, ear and wound infections.[12] Previous studies have assessed the *in vitro* efficacy of biocides through determination of minimum inhibitory concentrations (MICs) and/or minimum bactericidal concentrations.[15,27,33] Although the determination of MICs is important for the detection of efflux phenotypes (especially through detection of the ethidium bromide [EtBr] MICs) the *in vitro* efficacy of a biocide, as recommended by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), should involve the use of a neutralising agent or by the removal of the biocide.[25] This is important to avoid an over-estimation of the lethality of the biocide compound, since biocides are usually in contact with the bacteria only for a brief period of time.

To date, only a few studies have determined biocide susceptibility of *S. pseudintermedius*. This study compares the clonality, antimicrobial and biocide susceptibility of MSSP and MRSP that have been isolated from the nasal cavities of healthy animals as well as from infection sites.

Materials and Methods

Strain collection

Twenty MRSP and twenty MSSP strains isolated between 2007 and 2011 were included in the study. The isolates were collected at the Laboratory of Antimicrobial and Biocide Resistance, FMV-UTL, which receives samples from the Veterinary Teaching Hospital of FMV-UTL and private practices covering the area of the Lisbon region. Five isolates were from cats and 35 were from dogs. These included clinical infection (urinary tract infection, $n=6$; skin infection, $n=10$; ear infection, $n=5$; surgical site infection, $n=1$) and nasal colonization isolates ($n=18$).

Multi-locus sequence typing, spa and SCCmec typing

Isolates were characterized by Multi-locus sequence typing (MLST) using the MLST scheme of Bannoehr *et al.*,[2] which is based on five housekeeping genes (*pta*, *cpn60*, *tuf*, 16S rRNA and *agrD*),[24] and also by the newly described *S. pseudintermedius* MLST scheme, which is based on seven housekeeping genes (*ack*, *cpn60*, *fdh*, *pta*, *purA*, *sar*, *tuf*).[27] MRSP isolates were also characterized by *spa* and SCC*mec*-typing. *spa*-typing was performed by sequencing the polymorphic region of protein A gene (*spa*) and *spa* types were assigned according to previously proposed guidelines.[24] SCC*mec* types were determined using the multiplex PCR 1 and the multiplex PCR 2 according to Kondo and collaborators.[18] In multiplex PCR 1, the presence of *mecA* was confirmed and the *ccr* gene complex was determined. In multiplex PCR 2, the *mec* class complex was assessed.[18] The combination of the type *ccr* and *mec* complex was used to consign SCC*mec* types. *SCCmec* II-III was identified by PCR using primers described previously.[23]

eBURST analysis

Predicted lines of evolutionary descent in our collection of MRSP and MSSP isolates were identified using the eBURST algorithm (http://eburst.mlst.net). eBURST identified groups of related sequence types (ST) by assigning all members that shared identical alleles at four of the five gene loci (MLST-5 scheme) or six of the seven gene loci (MLST-7 scheme) with at least one other member of the group.[2] The founding ST of each group was determined by the ST with the greatest number of single locus variants (SLV).[28] Subgroups were defined by the existence of at least three SLV.

Pulsed-field gel electrophoresis

The *S. pseudintermedius* strains were compared for their genetic relatedness by *Sma*I macrorestriction, using a previously described protocol.[9] The *Sma*I fragment patterns were analysed with BioNumerics (Applied Maths, Kortrijk, Belgium), the similarities between profiles were calculated using the Dice coefficient with a maximum position tolerance of 1.0%. The patterns were clustered by using the unweighted pair group method with arithmetic averages based on a similarity cut-off value of 80%.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by the disk diffusion method and according to the Clinical and Laboratory Standards Institute guidelines.[7] The antibiotic resistance genes were detected using the custom-made microarray AMR+ve-2 (Alere GmbH, Cologne, Germany)[24] and by PCR.[19,24] Mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *grlA* were determined by PCR using the following primers: gyrA_pseudFW 5′-ATGAGTG TTATCGTATCTCGTGC-3′, gyrA_pseudRV 5′-GAACCGAA GTTACCTTGACCAT-3′, grlA_pseudFW 5′-AATACGTAT GATAAACATTTTCG-3′ and grlA_pseudRV 5′-TCGGTAT-CATCATAGTTCGG-3′, respectively. Mutations in the rifampicin resistance-determining region (RRDR) within the *rpoB* gene of the rifampicin-resistant isolates were amplified by PCR and sequenced as described previously.[17]

Biocide susceptibility

MICs were determined for the following antiseptics and dye: chlorhexidine acetate (CHA), benzalkonium chloride (BAC), triclosan (TCL) and EtBr. EtBr MIC determination is a simple screening procedure for identifying strains, which have increased expression of efflux pump genes or an efflux phenotype.[8] CHA, BAC and TCL were determined to further characterize any efflux phenotype. The bactericidal activity (at 5 minutes and 20°C) of four commercial dermatological preparations (Otodine®, Clorhexyderm Spot Gel®, Dermocanis Piocure-M® and Skingel®) was determined against MRSP and MSSP according to the document NF EN 1040–Essai quantitatif de suspension pour l'évaluation de l'activité bactericide de base des antiseptiques et des désinfectants chimiques.[1] Both Otodine and Clorhexyderm Spot Gel contain CHA (0.15% and 0.3%, respectively), Dermocanis Piocure-M has TCL (0.3%) and Skingel contains zinc oxide (10%). The full formulation of the biocide products can be found at the manufacture's website (www.icfpet.it). Briefly, isolates were grown on a solid medium for 24 hours at 37°C and suspended on a diluent to $1.5–5\times10^8$ colony-forming units/ml. Each cell suspension was inoculated into water containing the biocides and was exposed for 5 minutes, as recommended by NF1040.

To inactivate the biocides, the bacteria-biocide mixture was transferred into the neutralization medium (phosphate buffer 0.25 mmol/L pH 7.2) for 5 minutes. Then the mixture was inoculated onto Tryptone-Soy agar plates without the biocides. Bacteria growth was observed after incubation at 37°C for 24 hours. Bactericidal activity was defined as a logarithmical reduction on bacterial cell counts of at least five logarithms. *S. aureus* ATCC 29213 and *S. aureus* ATCC 6538 were used as quality controls. The detection of efflux genes *qacA/B*, *smr*, *qacG*, *qacH* and *qacJ* was performed by PCR.[3,8] The *qacA/B* positive amplicons were sequenced. The detection of *sh-fabI* was performed by PCR using primers described recently by Ciusa *et al.*, using *S. aureus* strain M0091 as a positive control.[6]

Results

Strain characterization by genotyping

The epidemiological, genotypic and phenotypic traits of the forty MRSP and MSSP isolates under study are shown in Table 1. MLST-7 allowed a better discrimination than MLST-5 and further distinguished among strains (Table 1). The MSSP strains were divided into 19 or 24 different STs according to the MLST-5 and MLST-7 schemes, respectively (see Table 1). Two novel *cpn60* alleles (alleles 43 and 44, accession numbers JX976294 and JX982108, respectively) and four novel *pta* alleles (alleles 32, 34, 35 and 36, accession numbers JX982110, KC438371, JX982112 and JX987962, respectively) were found. Using the MLST-5 scheme, 17 MRSP belonged to ST71, two belonged to ST97 and one to ST2 (Fig. 1a). When applying the new MLST-7 scheme only 14 MRSP ST71 isolates were assigned to the ST71, and three being assigned to ST203; two ST97 were subdivided into ST196 and ST213, and ST2 was assigned to ST195. Yet ST203 and ST195 belonged to the clonal complex (CC) 71, as detected by the eBURST analysis (Fig. 1b). Likewise ST196 and ST213 differed only by one allele and belonged to CC196 (Fig. 1b). Pulsed-field gel electrophoresis (PFGE) analysis, based on a similarity cut-off value of 80%, revealed two major clusters of MRSP, one containing the CC71 isolates and the other cluster having the CC196 strains (Fig. 2). The MRSP isolate ST195 was non-typeable by SmaI restriction PFGE. Similar to MLST results, PFGE analysis revealed that the MSSP isolates were genetically more diverse (Fig. 3). eBURST analysis performed in our collection of MRSP and MSSP isolates was very different when using the MLST-5 and MLST-7 schemes. When applying the MLST-5 scheme eBURST showed that the *S. pseudintermedius* isolates belonged to very similar STs, only differing in one or two of the five loci examined (Fig. 3a). As expected, eBURST analysis using the MLST-7 scheme had very different results, with only a few STs relating with another (Fig. 1b) and the MSSP being singletons (data not shown).

Antimicrobial susceptibility and resistance genes

Antimicrobial resistance patterns of the *S. pseudintermedius* isolates are shown in Table 1. All MRSP isolates were resistant to erythromycin, clindamycin, fluoroquinolones (ciprofloxacin, enrofloxacin, moxifloxacin, norfloxacin, ofloxacin and pradofloxacin), trimethoprim/sulfamethoxazole, gentamicin, tobramycin, kanamycin and streptomycin. Additionally 17 MRSP isolates had tetracycline-resistance, one had chloramphenicol resistance and one was resistant to rifampicin. MSSP were more susceptible than MRSP strains to the tested antibiotics. Eight strains were susceptible to all antibiotics. All isolates were susceptible to fluoroquinolones. Ampicillin and penicillin resistance was present in nine MSSP strains, while eight were resistant to tetracycline. Two MSSP strains were resistant to erythromycin, clindamycin, kanamycin, streptomycin and chloramphenicol and one was resistant to trimethoprim/sulfamethoxazole. Resistances were attributed to the presence of the penicillin binding protein gene *mecA* (all β-lactams), the β-lactamase gene *blaZ* (penicillin), the tetracycline resistance genes *tet*(M) and *tet*(K), aminoglycoside acetyltransferase and phosphotransferase gene *aac(6′)-Ie–aph(2′)-Ia* (all aminoglycosides except streptomycin), phosphotransferase gene *aph(3′)-III* (kanamycin, neomycin, paromomycin, amikacin, gentamicin B), streptomycin adenyltransferase gene *ant(6)-Ia*, the macrolide, lincosamide and streptogramin B 23S rRNA methylase gene *erm*(B), the lincosamide nucleotidyltransferase *lnu*(A), the chloramphenicol acetyltransferase gene cat_{pC221}, the trimethoprim-resistance dihydrofolate reductase gene *dfr*(G). Two amino acid substitutions (S84L of GyrA and S80I of GrlA) were found in the QRDR of fluoroquinolone-resistant isolates. The rifampicin-resistant isolate had an A522D substitution in the RRDR. One MSSP strain harboured a *lnu*(A) gene but was not resistant to clindamycin.

Biocide susceptibility, qac *and* sh-fabI *genes*

Fourteen and six MRSP isolates presented an MIC of 1 mg/L and 2 mg/L of BAC, respectively (Table 2). Eighteen MSSP isolates had an MIC of 0.5 mg/L to BAC (Table 2). All MRSP and MSSP strains had an MIC of 1 mg/L to CHA. All MRSP isolates and 18 MSSP had an MIC to TCL of ≤0.003 mg/L, while one isolate had an MIC of 0.125 mg/L. None of the isolates carried the recently described TCL resistance gene *sh-fabI*. MIC to EtBr were ≤4 mg/L, except for one isolate, which showed an EtBr MIC of 32 mg/L (Table 2). This MSSP isolate (FMV20A/08) had an MIC of four to BAC and harboured the quaternary compound resistance gene *qacA*. Another MSSP isolate (FMV750/10) had the *qacB* gene but no detectable efflux mechanisms. All MRSP isolates were negative for the efflux genes tested.

Three preparations, Otodine, Clorexyderm Spot Gel and Dermocanis Piocure-M, had bactericidal activity against all MRSP and MSSP isolates. However, Skingel could not achieve a five log reduction of the bacterial count.

Discussion

Methicillin resistance have only been recently reported in *S. pseudintermedius* strains, but their capacity to resist to antimicrobial therapy is already a worldwide concern.[23,30] ST71 was the predominant clone emphasizing its spread. The use of the new MLST scheme based on seven housekeeping genes allowed distinguishing between some of the strains of the CC71, revealing new ST195 and ST203. ST71 has been previously described among MRSP colonization isolates from dogs in Portugal;[9] however, this is the first report of MRSP ST196 and ST213 (CC196), which are not related to CC71. MSSP isolates, instead, were more genetically diverse, with all MSSP isolates corresponding to a single ST. These findings are in agreement with two previous reports,[2,4] where MRSP isolates were restricted to a small number of ST, while MSSP strains revealed substantial clonal diversity.

Table 1. Epidemiological, Genotypic, and Phenotypic Traits of the Forty Strains Included in the Study

Strains	*Sample source*	*Animal clinical status*	*ST based on 5-MLST*	*ST based on 7-MLST*	spa *type*	*SCCmec type*	*Antimicrobial resistance phenotype*
FMV1879B/07	Cat	Urinary tract infection	97	196	t06	V	OXA-PEN-AMP-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV5/08	Dog	Healthy	97	213	t06	V	OXA-PEN-AMP-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV29/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV34C/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV34D/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV67/08	Dog	Healthy	71	203	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV71/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV104/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV116/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV178/09	Dog	Healthy	71	203	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV4877/10	Dog	Pyoderma	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMVP61/ZP	Dog	Pyoderma	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV3891/09	Cat	Urinary tract infection	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV1860/10	Cat	Otitis	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV3008/10	Dog	Otitis	71	203	t06	II-III	OXA-PEN-AMP-ERY-CLI-GEN-TOB-KAN-STR-SXT-CIP-FQ
FMV3607/10	Cat	Urinary tract infection	71	71	t06	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ-CHL
FMV5819/10	Dog	Pyoderma	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ-RIF
FMVStaph4	Dog	Pyoderma	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV981/11	Dog	Pyoderma	71	71	t05	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV13/2011	Dog	Discospondylitis	2	195	t02	II-III	OXA-PEN-AMP-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV5699/07	Dog	Urinary tract infection	169	207	–	–	–
FMV9/08	Dog	Healthy	40	17	–	–	–
FMV12/08	Dog	Healthy	52	216	–	–	PEN-AMP-ERY-CLI-KAN-STR-CHL
FMV15/08	Dog	Healthy	54	210	–	–	–
FMV20A/08	Dog	Healthy	93	211	–	–	PEN-AMP
FMV33/08	Dog	Healthy	192	201	–	–	–
FMV41/08	Dog	Healthy	176	212	–	–	TET
FMV52/08	Dog	Healthy	136	217	–	–	–
FMV66/08	Dog	Healthy	171	214	–	–	TET
FMV76/08	Dog	Healthy	177	215	–	–	PEN-AMP-TET
FMV750/10	Dog	Pyoderma	29	200	–	–	PEN-AMP-TET
FMV2944/10	Dog	Pyoderma	17	202	–	–	PEN-AMP-TET
FMV635/10	Dog	Urinary tract infection	170	209	–	–	PEN-AMP-TET
FMV3413/09	Dog	Otitis	20	204	–	–	TET
FMV2183/10	Dog	Otitis	172	197	–	–	–
FMV2999/10	Dog	Urinary tract infection	40	199	–	–	–
FMV2218/10	Dog	Pyoderma	44	198	–	–	–
FMV5386/10	Dog	Pyoderma	174	206	–	–	PEN-AMP
FMV6098/10	Dog	Pyoderma	175	208	–	–	PEN-AMP-SXT
FMV4778/09	Cat	Otitis	178	205	–	–	PEN-AMP-TET-ERY-CLI-KAN-STR-CHL

AMP, ampicillin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; FQ, fluoroquinolones, including ciprofloxacin, enrofloxacin, moxifloxacin, norfloxacin, ofloxacin, and pradofloxacin; GEN, gentamicin; KAN, kanamycin; OXA, oxacillin; PEN, penicillin; RIF, rifampicin; TET, tetracycline; TOB, tobramycin; ST, sequence types; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim.

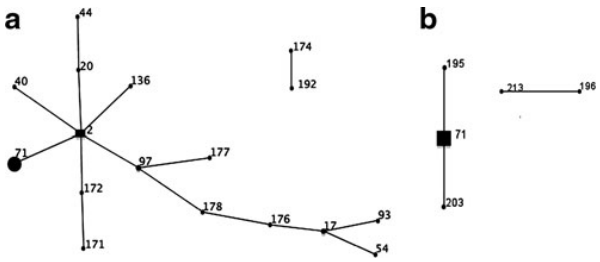

FIG. 1. Schematic diagram of the clonal relatedness of *Staphylococcus pseudintermedius* sequence types (ST) predicted by eBURST analysis using the multilocus sequence typing (MLST)-5 **(a)** and the MLST-7 **(b)** schemes, respectively. Each black dot represents an ST and the dot size is proportional to the number of isolates of that ST. The square corresponds to the predicted group founder and ST97 and ST17 represent predicted subgroup founders **(a)**. Single-locus variants are linked by lines. Methicillin-susceptible *S. pseudintermedius* (MSSP) singletons are not shown in this figure.

However, two recent reports found the *mecA* gene in a considerable high number of ST,[11,22] indicating that different lineages of *S. pseudintermedius* can acquire SCC*mec* elements. Only two types of SCC*mec* elements were detected among the MRSP from Portugal, namely SCC*mec* II-III in isolates of CC71 and SCC*mec* V in isolates of CC196.

Antimicrobial resistance is typically very different between MRSP and MSSP. While MRSP tend to be multidrug-resistant, MSSP are usually only resistant to ampicillin and penicillin, due to the presence of the *blaZ* gene.[12] Accordingly we found a multidrug resistant pattern in all MRSP isolates. However, two MSSP strains were also resistant to more than three antimicrobial classes, categorizing these strains as multidrug-resistant. Some studies have also identified multidrug-resistance among MSSP isolates.[13,14,29] Nevertheless, in the majority of the studies MSSP strains were only resistant to ampicillin and one additional antimicrobial class.[12,30] Several antimicrobial resistance genes have been detected in *S. pseudintermedius* strains[16] and our strains exhibit the same genes as detected before. Contrary to the study of Vanni and colleagues,[31] which only detected resistance to second- and third-generation fluoroquinolones in *S. pseudintermedius* isolates, our fluoroquinolone-resistant *S. pseudintermedius* strains were resistant to second (ciprofloxacin, enrofloxacin, norfloxacin, and ofloxacin), third generation (pradofloxacin), and fourth generation (moxifloxacin) fluoroquinolones. The same authors argued that a single alteration in *grlA* would be sufficient to confer resistance against older fluoroquinolones but an additional mutation in *gyrA* was required for resistance to new fluoroquinolones to develop, as it occurs in *S. aureus* and coagulase-negative staphylococci isolates.[31] Accordingly, our strains presented resistance to all the fluoroquinolones tested, including moxifloxacin, due to the presence of mutations at both the *gyrA* and *grlA* genes.

Surprisingly there was a major difference between the mechanisms of resistance to tetracycline: *tet*(K) genes were only identified among MRSP strains, while *tet*(M) was only found among MSSP isolates. The *tet*(K) gene codes for an efflux pump of the major facilitator superfamily and is usually found on small plasmids.[16] In contrast, *tet*(M) codes for ribosome protective proteins and has been identified as part of conjugative transposons, such as Tn*916* and Tn*1545*.[16] Other studies have identified *tet*(M) in other MRSP strains, yet in MRSP ST71 only *tet*(K) has been detected, which could indicate that this clone has a preference for plasmid-borne tetracycline resistance rather than *tet* transposon-borne genes.

Rifampicin resistance in MRSP isolates has been described previously.[17] Mutation at *rpoB* codon 522 was identified in a clinical isolate after treatment of a clinical infection with a combination of rifampicin and tetracycline.[17] Our rifampicin-

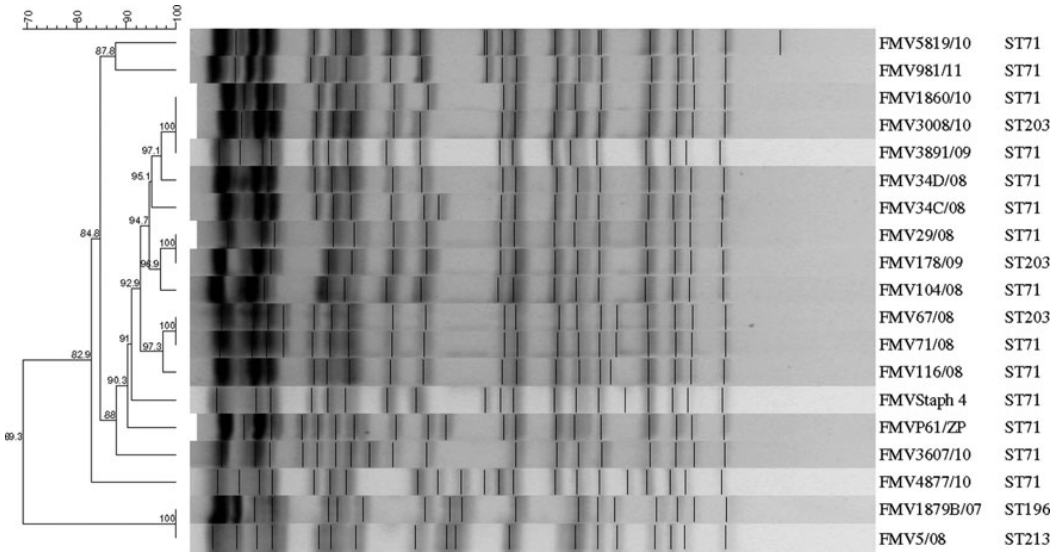

FIG. 2. Dendogram of chromosomal DNA digested with SmaI of methicillin-resistant *S. pseudintermedius* strains and relatedness to ST. Pulsed-field cluster determination using a Dice similarity coefficient with an optimization of 1% and a band tolerance setting of 1%.

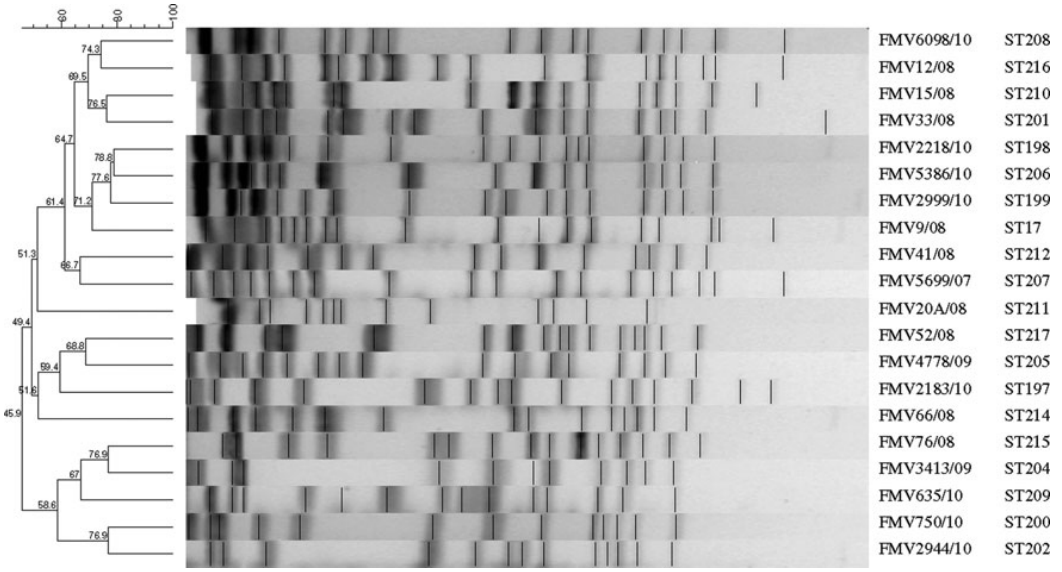

FIG. 3. Dendogram of chromosomal DNA digested with SmaI of MSSP strains and relatedness to ST. Pulsed-field cluster determination using a Dice similarity coefficient with an optimization of 1% and a band tolerance setting of 1%.

resistant MRSP isolate came from a dog with pyoderma, with no previous recorded history of rifampicin treatment. As stated before,[17] the observed *rpoB* mutation could have occurred spontaneously or the isolate could have been transferred from a previously treated dog.

Since the discovery of multidrug-resistant *S. pseudintermedius* there has been an increasing interest in additional bactericidal therapeutics, other than the use of antibiotics. Susceptibility to biocides has now become an urgent matter.

Table 2. MICs of Dyes (Ethidium Bromide) and Biocides (Benzalkonium Chloride, Chlorhexidine Acetate and Triclosan), and Genes Associated with Efflux Phenotype

	MICs (mg/L)				
Number of strains	*BAC*	*CHA*	*EtBr*	*TCL*	*Efflux genes*
MRSP					
10	1	1	2	≤0.003	–
3	2	1	4	≤0.003	–
3	2	1	2	≤0.003	–
2	1	1	4	≤0.003	–
1	1	1	1	0.007	–
1	1	1	2	0.007	–
MSSP					
11	0.5	1	1	≤0.003	–
6	0.5	1	2	≤0.003	
1	4	1	32	0.125	*qacA*
1	0.5	1	2	≤0.003	*qacB*
1	1	1	2	4	–
S. aureus ATCC 6538	1	1	8	≤0.003	–

BAC, benzalkonium chloride; CHA, clorhexidine acetate; EtBr, ethidium bromide; MICs, minimum inhibitory concentrations; MRSP, methicillin-resistant *Staphylococcus pseudintermedius*; MSSP, methicillin-resistant *S. pseudintermedius*; TCL, triclosan.

In this study, we compared the *in vitro* efficacy of four commercial biocides using the methodology recommended by the SCENIHR.[25] At the same time we performed the determination of the biocide MICs to detect decreased susceptibility related to efflux activity. Only one strain showed higher EtBr MIC values compared to the wild-type *S. aureus* ATCC6538 and harboured a *qacA* gene. The *qacB*-positive MSSP strain (FMV750/10) did not show any decreased susceptibility related to efflux activity. The same strain also had a *lnu*(A) gene but was not clindamycin-resistant. This could indicate a failure in the regulation and/or induction mechanism of these genes. However, further studies are needed to address this issue. To the best of our knowledge, this is the first description of *qacA* and *qacB* genes among *S. pseudintermedius* strains.

The efficacy of chlorhexidine has been previously tested *in vitro* and also *in vivo*. In our study we found an MIC value of 1 mg/L for all MRSP and MSSP strains, which is lower than the MIC range found by Valentine and colleagues[29] (4–16 mg/L) but within the range found by Murayama *et al.*, (0.5–1 mg/L).[20] This latter study could not detect any *qacA/B* or *smr* genes. This MIC of 1 mg/L is lower than the clinically used concentrations and so it is not surprising that Otodine® and Clorexyderm Spot Gel® were efficient at killing the MSSP and MRSP strains. There was no difference in the efficacy of the chlorhexidine products, but previous studies have suggested that products with higher concentrations of CHA (3%–4%) were more effective than products with a lower concentration (2%–2.5%).[15,33] Still, an *in vivo* study comparing the use of two different chlorhexidine formulations (CHA 2% and chlorhexidine gluconate 4%) for the treatment of cephalexin-resistant *S. pseudintermedius* pyoderma found no differences in the efficacy of the two shampoos.[21]

QAC efflux pumps are known to extrude BAC;[8] however, the MICs previously found in other studies are still below the typically used concentrations of 10 g/L.[29] In our study we

detected two strains harbouring *qac* genes but their MICs were also below the in-use concentration. However, even if the strains appeared susceptible *in vitro* in the presence of these genes, they may challenge biocide therapy *in vivo*.

None of the strains with high MIC to TCL carried the newly described plasmid-mediated TCL resistance gene *sh-fabI*.[6] The higher MIC values in the two MSSP strains, and the absence of the *sh-fabI* gene may indicate that another mechanism is present, probably mutations in the original *fabI* gene, which have been previously described in *S. aureus* and *S. haemolyticus* strains.[6] A recent study assessed the MIC of TCL against MRSP and MSSP strains.[29] The authors concluded that TCL demonstrated excellent activity against all bacterial isolates with a MIC ≤ 0.5 mg/L.[29] In this study, we detected one MSSP strain with a MIC of 4 mg/L to TCL, which is higher compared to the wild-type *S. aureus* ATCC6538 (MIC ≤ 0.003) and the other *S. pseudintermedius* strains. However, when testing the bactericidal activity of Dermocanis Piocure-M,® a commercial product with a TCL concentration 750 times higher than the MIC, no bacterial growth was observed. Likewise, the MSSP strain presenting an efflux phenotype, when challenged with three commercial products containing biocides was also not able to survive. This could mean that although some strains have efflux mechanisms to biocides, they will not be able to survive if the biocides are used at the correct concentration and exposure time.

Skingel® is an antiphlogistic product, containing zinc oxide, which is known to have antibacterial properties.[26] Zinc oxide has been shown to reduce *S. aureus* viability and biofilm formation when incorporated as a nanoparticle into films of polyvinyl chloride (endotracheal tubes and catheters).[26] However, Skingel was not able to achieve a five-log reduction in the bacterial cell number and so had no bactericidal effect on *S. pseudintermedius* strains. Zinc resistance has been detected in *S. aureus* strains of animal origin and has been strongly associated with methicillin resistance.[5] Further studies are needed to evaluate heavy metal resistance in *S. pseudintermedius*.

S. pseudintermedius have become a serious therapeutic challenge and new MRSP lineages are emerging in several countries, including Portugal. Although multidrug-resistance is more common in methicillin-resistant strains, some of the methicillin-susceptible strains also exhibited multidrug-resistance profile. The use of biocides, like CHA and TCL, seems to be a clinically effective and a safe topical therapeutic option.

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Disclosure Statement

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3.2.3 Trends in antimicrobial resistance in clinical staphylococci isolated from companion animals over a 16-year period

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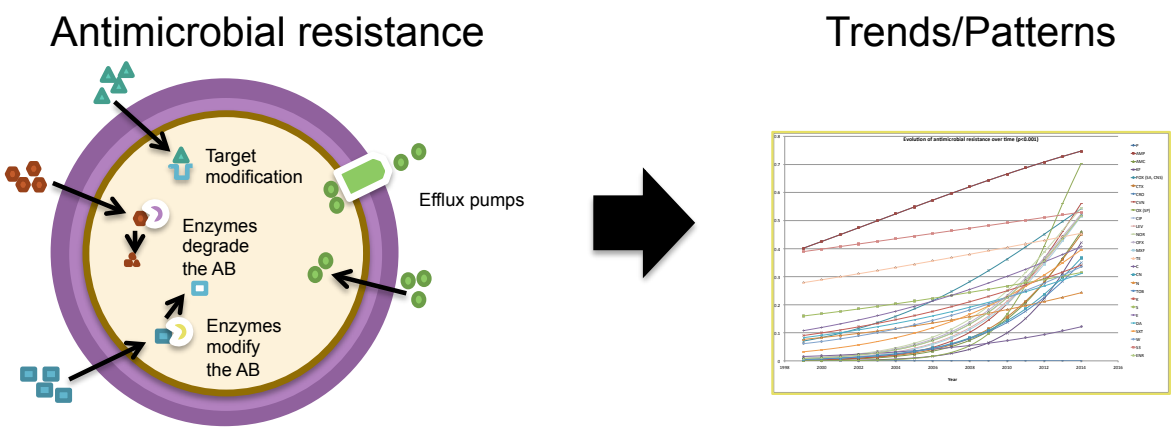

Trends and molecular mechanisms of antimicrobial resistance in clinical staphylococci isolated from companion animals over a 16-year period

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Objectives: To investigate the evolution of resistance to antimicrobials, corresponding mechanisms and molecular characteristics of *Staphylococcus* spp, between 1999-2014.
Methods: Susceptibility for 38 antimicrobials were determined in 632 clinical staphylococcal isolates obtained from companion animals (dogs, cats, horses and other animals). Twenty antimicrobial resistance genes, including *mecA* and *mecC*, were screened by PCR. Methicillin-resistant staphylococci (MRS) were characterized by *spa* (*S. aureus*), SCC*mec*, MLST and PFGE typing. Statistical analyses were performed using SAS v9.3 and differences were considered relevant if P≤0.05.
Results: The *mecA* gene was identified in 74 staphylococcal isolates (11.7%): 11 *S. aureus* (MRSA, 40.7%), 40 *S. pseudintermedius* (MRSP, 8.7%) and 23 coagulase-negative staphylococci (MRCoNS, 26.7%). Resistance to the majority of antimicrobials and the number of *mecA*-positive isolates increased significantly over time. Eighteen *spa* types were identified, including two new ones. MRSA isolates were divided into 3 PFGE clusters that included ST22-IV, ST105-II, ST398-V and ST5-VI. Most methicillin-resistant *S. epidermidis* isolates were of CC5, including a new ST, and clustered in 8 PFGE clusters. MRSP were grouped into 5 PFGE clusters and included ST45-nt, ST71-II-III, ST195-III, ST196-V, ST339-nt, ST342-IV and the new ST400-III. Methicillin-resistant *S. haemolyticus* clustered in two PFGE clusters.
Conclusions: The significant increase of antimicrobial-resistant and *mecA*-positive isolates in the last years is worrying. Furthermore, several isolates are multidrug-resistant, which complicates antimicrobial treatment and raises the risk of transfer to humans or human isolates. Several clonal lineages of MRSA and MRSE circulating in human hospitals and in the community were found, suggesting that companion animals can become infected with and contribute to the dissemination of highly successful human clones. Urgent measures, like determination of clinical breakpoints and guidelines for antimicrobial use are urgently needed.

Keywords: *mecA*, staphylococci, resistance genes, antimicrobials

Introduction

Staphylococci are a group of bacteria with clinical, veterinary, agricultural, and economic importance because of their wide range of virulence factors and ability to become resistant to antimicrobials. This feature should be considered and antimicrobial susceptibility testing (AST) is important to monitor the spread of antimicrobial-resistant staphylococci.[1] Therefore, monitoring programs may help uncover new resistance trends and evaluate the usefulness of the available antimicrobials against staphylococci. Companion animals, in particular, are

frequently treated with the same antimicrobial classes that are used in human medicine.[2,3]

The genus *Staphylococcus* causes a different array of infections, and the most common species in companion animal practice are the coagulase-positive *Staphylococcus pseudintermedius* (formerly called *S. intermedius*), *S. schleiferi* and *S. aureus.* These are mostly found in skin samples, ear samples and as the cause of urinary tract infections (UTI).[1] CoNS, on the other hand, are usually not considered pathogenic but they are often considered reservoirs of antimicrobial resistance genes, like the *mecA* gene.[4] Presence of the *mecA* or *mecC* genes, is one of the most significant features encountered in staphylococcal species. These genes mediate resistance to ß-lactams, which are first-line antimicrobial choices for the treatment of infections in human and veterinary medicine and are considered by the WHO as "critically-important" antimicrobials.[5] Furthermore, knowledge on the genotype of such isolates is important to assess the risk of transfer of methicillin-resistant staphylococcal (MRS) isolates between companion animals and humans.

The main objective of this study was to investigate the trends in antimicrobial resistance in clinical staphylococci isolated from companion animals over a 16-year period (1999-2014). Furthermore, we identified the genetic mechanisms underlying the antimicrobial resistance. Finally, we characterized the genotype of the MRS to understand evolutionary steps driving the spread of these isolates in companion animals.

Materials and methods

Isolate collection

Six hundred and thirty-two staphylococcal isolates isolated from companion animals between 1999 and 2014 were included in the study. The isolates were collected at the Clinical Laboratory (CL) and at the Antimicrobial and Biocide Resistance Laboratory (ABRL), FMV-UL, which receive samples from the Veterinary Teaching Hospital of FMV-UL and private practices throughout the Lisbon region. The isolates were obtained from clinical infections and sent to the laboratory along with a small form with animal data, such as species, breed, age and sex, clinical description of the sample site and suspected pathology course. Each isolate was considered individually, and cases where more than one isolate originated from the same animal (i.e., different staphylococci isolated at the same time from the same specimen or at different sampling times) were considered only if the staphylococcal species or genotype differed between isolates.

Staphylococcal species identification

Both the CL and ABRL laboratories use phenotypical tests (BD™ BBL™ Crystal Gram Positive ID Kit; Becton, Dickinson and Company, Maryland, USA) to determine the staphylococcal species. All species were confirmed by PCR (*S. epidermidis*-specific primers Se705-1, Se705-2; *S. saprophyti-*

cus-specific primers Sap1, Sap2; *S. xylosus*-specific primers XYL F, XYL R; *S. simulans*-specific primers SimF, SimR) [6-10] and/or sequencing of 16S rRNA gene. The sequences were then compared using the nucleotide basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/).

Antimicrobial susceptibility testing

All isolates were tested by disk diffusion according to CLSI standards and *S. aureus* ATCC29213 was used for quality control purposes, whenever a new antimicrobial batch was used.[11] A total of 38 antimicrobials (Oxoid, Hampshire, United Kingdom) were tested: amikacin (AMK, 30 µg), ampicillin (AMP, 10 µg), amoxicillin/clavulanic acid (AMC, 30 µg), cefalotin (CEF, 30 µg), cefotaxime (CTX, 30 µg), cefovecin (CVN, 30 µg), cefoxitin (FOX, 30 µg), ceftriaxone (CRO, 30 µg), chloramphenicol (CHL, 30 µg), ciprofloxacin (CIP, 5 µg), clindamycin (CLI, 2 µg), enrofloxacin (ENR, 5 µg), erythromycin (ERY, 15 µg), florfenicol (FFC, 30 µg), fusidic acid (FUS, 10 µg), gentamicin (GEN, 10 µg), kanamycin (KAN, 30 µg), levofloxacin (LEV, 5 µg), linezolid (LZD, 30 µg), moxifloxacin (MXF, 5 µg), mupirocin (MUP, 5 µg), neomycin (NEO, 30 µg), netilmicin (NET, 30 µg), nitrofurantoin (NIT, 300 µg), norfloxacin (NOR, 10 µg), ofloxacin (OFX, 5 µg), oxacillin (OXA, 1 µg), penicillin G (PEN, 10 units), quinupristin/dalfopristin (Q/D, 15 µg), rifampicin (RIF, 5µg), streptomycin (STR, 15 µg), sulphonamides (SUL, 300 µg), teicoplanin (TEC, 30 µg), tetracycline (TET, 30 µg), tobramycin (TOB, 10 µg), trimethoprim (TMP, 5 µg), trimethoprim/sulfamethoxazole (SXT, 25 µg) and vancomycin (VAN, 30 µg). Some antimicrobials were included since they are clinically relevant antimicrobial agents and others were included for antimicrobial resistance epidemiology purposes (like linezolid, quinupristin/dalfopristin, teicoplanin). Results were interpreted according to CLSI VET01-S2[12] (oxacillin for *S. pseudintermedius*, enrofloxacin, gentamicin, clindamycin), CLSI M100-S24[13] (ampicillin, penicillin, cefoxitin for *S. aureus* and CoNS, teicoplanin, amikacin, kanamycin, netilmicin, tobramycin, erythromycin, tetracycline, ciprofloxacin, levofloxacin, norfloxacin, ofloxacin, moxifloxacin, nitrofurantoin, trimethoprim/sulfamethoxazole, sulphonamides, trimethoprim, chloramphenicol, rifampin, quinupristin/dalfopristin, linezolid), CA-SFM VET-10[14] (neomycin) and CA-SFM 10[15] (streptomycin, mupirocin). The EUCAST guidelines[16] were used for fusidic acid interpretation. Breakpoints for amoxicillin/clavulanic acid, cephalosporins and vancomycin were recently removed from CLSI. However, as these antimicrobials were included in the susceptibility panel of both laboratories, we used the breakpoints given by the last CLSI containing them (CLSI M100-S16[17] and CLSI M100-S22[18]). The breakpoints for cefovecin were retrieved from the manufacturer (S≥24; I 21-23; R≤20). There are no breakpoints for florfenicol against staphylococci and we assessed the distribution of the zone diameters detected in our study (Supplementary

Figure 1), estimating a resistance breakpoint of R<19. A bacterial isolate was considered multidrug-resistant (MDR) when it exhibited resistance to three or more antimicrobial classes.[19] Isolates with intermediate susceptibility were regarded as susceptible.

Detection of antimicrobial resistance genes

The presence of the *mecA* and *mecC* genes was tested in all staphylococcal isolates. Other antimicrobial resistance genes were investigated only when phenotypical resistance was observed. Genes previously reported for resistance to ß-lactams (*blaZ*), aminoglycosides [*aadE*, *aadD*, *aphA3, aacA-aphD*], macrolides/lincosamides [*erm*(A), *erm*(B), *erm*(C), *msrA*, *mph*(C)], tetracyclines [*tet*(M), *tet*(K)], fusidic acid [*fusB, fusC*], chloramphenicol (*cat* pC221), florfenicol (*fexA*) and trimethoprim [*dfr*(G), *dfr*(K)] were detected by PCR.[20,21]

Molecular characterization

All *S. aureus* isolates were subjected to *spa* typing and *spa* types were assigned through the Ridom web server (http://www.ridom.de/spaserver/). Multilocus sequence typing (MLST) was performed in the methicillin-resistant *S. aureus* (MRSA), *S. pseudintermedius* (MRSP, representative isolates determined by PFGE) and *S. epidermidis* (MRSE) isolates (http://www.mlst.net/databases/default.asp; http://pubmlst.org/databases/). The SCC*mec* types were determined as described previously.[22] The MRS isolates were compared by SmaI PFGE, using previously described protocols.[21-23] PFGE clusters were defined when the isolates had ≥80% similarity.

Statistical analyses

Statistical analyses were performed with SAS software version 9.3 (SAS Institute Inc., Cary, N.C.), and results were considered significant when p≤0.05. For the purpose of statistical analyses, we defined staphylococcal species as: *S. aureus*, *S. pseudintermedius*, *S. schleiferi* and CoNS. The association between staphylococcal species, antimicrobial resistance and resistance genes was assessed using either the chi-squared test or Fisher exact test (when n≤5).

The importance of animal species (dog or cat), type of infection (pyoderma, urinary tract infection [UTI] or otitis) and age within animal species, as possible risk factors of resistance to the different antimicrobials was analysed by logistic regression, considering each factor individually.

The evolution over time of the proportion of isolates resistant to different antimicrobials was analysed by logistic regression, using year as independent variable. This test was also used to determine if there was a significant increase in the proportion of *mecA*-positive isolates over time.

This research study involved a total of 632 staphylococcal isolates from 614 animals, of which 537 isolates were from dogs (84.9%), 80 from cats (12.7%), 10 from horses

(1.6%), 5 from other animals (0.8%). Overall, 252 isolates were from females (39.8%), 346 from males (54.7%) and 34 were unknown (5.4%). The average age was of 6.8 years for dogs, 5.5 for cats and 11.4 for horses.

Isolates were most frequently isolated from otitis (307 isolates, 48.6%), followed by 178 isolates from pyoderma (28.2%), 90 from UTI (14.2%), 10 from surgical site infections (1.6%) and 47 from other types of infection (7.4%). The frequency of each staphylococcal species is shown in Table 1, with a clear predominance of *S. pseudintermedius*, with 446 isolates (70.6%), followed by CoNS species with 86 isolates (13.6%), *S. schleiferi* with 73 isolates (11.6%) and 27 isolates of *S. aureus* (4.3%).

Table 1. Staphylococcal species distribution and frequency of the *mecA* gene.

Staphylococcal species	Frequency (%)	Frequency of the *mecA* gene (%)
S. aureus	27 (4.3)	11 (40.7)
S. caprae	1 (0.2)	1 (100)
S. cohnii	2 (0.3)	0 (0.0)
S. epidermidis	20 (3.2)	11 (55.0)
S. felis	26 (4.1)	0 (0.0)
S. haemolyticus	13 (2.1)	8 (61.5)
S. hominis	1 (0.2)	1 (100)
S. kloosi	1 (0.2)	0 (0.0)
S. lentus	2 (0.3)	1 (50.0)
S. lugdunensis	1 (0.2)	0 (0.0)
S. pseudintermedius	446 (70.6)	40 (8.7)
S. saprophyticus	4 (0.6)	0 (0.0)
S. schleiferi	73 (11.6)	0 (0.0)
S. simulans	9 (1.4)	1 (11.1)
S. warneri	3 (0.5)	0 (0.0)
S. xylosus	3 (0.5)	0 (0.0)

The frequencies of antimicrobial resistance and antimicrobial resistance genes are shown in Tables 2 and 3, respectively. All isolates were susceptible to vancomycin, teicoplanin, linezolid, netilmicin and quinupristin-dalfopristin. About 35% of the staphylococci isolates were multidrug-resistant. The *mecA* gene was identified in 74 staphylococci isolates (11.7%): 11 *S. aureus* (40.7%), 40 *S. pseudintermedius* (8.7%) and 23 CoNS (26.7%) (Table 1). None of the isolates carried the *mecC* gene. We detected the *fexA* gene in 3 isolates that were resistant to florfenicol and chloramphenicol (1 *S. aureus* and 2 *S. pseudintermedius*). In these *fexA*-positive isolates we searched for the *cfr* gene, and we detected for the first time the *cfr* gene in a *S. pseudintermedius* isolate (confirmed by sequencing).

The CoNS and *S. aureus* isolates had higher probabilities of having the *mecA* gene ($p<0.05$) than *S. pseudintermedius* or *S. schleiferi* (40.7 and 26.7 versus 8.7% and 0%, respectively; Table 3). Resistance to ampicillin/penicillin and the presence of the *blaZ* gene were highly associated with both *S. aureus* and *S. pseudintermedius* ($p<0.001$; Tables 2 and 3, respectively). *S. aureus* were more resistant to fluoroquinolones [enrofloxacin ($p<0.03$), ciprofloxacin ($p<0.03$), levofloxacin ($p<0.005$), norfloxacin ($p<0.04$), moxifloxacin ($p<0.002$)] than the other species (Table 2). The *S. pseudintermedius* were more likely ($p<0.05$) to have the *erm*(B) gene and less likely ($p<0.05$) to have the *erm*(C) gene than any other species (Table 3). The streptomycin-resistance was more associated with *S. pseudintermedius* ($p<0.02$; Table 2) and the *aadE* gene was actually only present in this species (Table 3). On the other hand, tetracycline-resistance was associated with CoNS ($p<0.02$) and *S. pseudintermedius* ($p<0.0003$; Table 2), with the *tet*(K) gene more associated with CoNS ($p<0.006$), and the *tet*(M) gene with *S. pseudintermedius* ($p<0.0001$; Table 3). Resistance to fusidic acid was higher in the CoNS isolates ($p<0.02$; Table 2).

Table 2. Frequency of antimicrobial resistance for the total isolates, and per staphylococcal strain. The p-value refers to the association between antimicrobial resistance and staphylococcal species.

Antimicrobial	Percentage of resistance in all strains (CI)	Percentage of resistance in coagulase-negative staphylococci	Percentage of resistance in *S. aureus*	Percentage of resistance in *S. pseudintermedius*	Percentage of resistance in *S. schleiferi*	p-value
Ampicillin	58.7 [54.7-62.6]	40.7	77.8	64.4	38.4	<0.0001
Penicillin	58.7 [54.7-62.6]	40.7	77.8	64.4	38.4	<0.0001
Amoxicillin/clavulanic acid	7.8 [5.8-10.1]	5.8	37.0	7.6	0.0	<0.0001
Cephalexin	6.0 [4.3-8.2]	2.3	22.2	6.7	0.0	0.0002
Cefovecin	10.4 [8.2-13.1]	19.8	40.7	8.5	0.0	<0.0001
Ceftriaxone	8.5 [6.5-11.0]	11.6	37.0	7.6	0.0	<0.0001
Cefotaxime	8.5 [6.5-11.0]	10.5	37.0	7.9	0.0	<0.0001
Cefoxitin[a]	28.3 [20.2-37.6]	24.4	40.7	-	-	0.1005
Oxacillin[b]	8.7 [6.3-11.8]	-	-	8.7	-	-
Enrofloxacin	12.3 [9.9-15.2]	14.0	40.7	9.2	19.2	<0.0001
Ciprofloxacin	12.3 [9.9-15.2]	14.0	40.7	9.2	19.2	<0.0001
Levofloxacin	11.4 [9.0-14.1]	14.0	40.7	9.0	12.3	<0.0001
Norfloxacin	12.7 [10.2-15.5]	16.3	40.7	9.2	19.2	<0.0001
Ofloxacin	12.8 [10.3-15.7]	16.3	40.7	9.2	20.6	<0.0001
Moxifloxacin	10.1 [7.9-12.8]	11.6	40.7	8.1	9.6	<0.0001
Tetracycline	34.8 [31.1-38.9]	23.3	3.7	44.0	4.1	<0.0001
Nitrofurantoin	0.5 [0.1-1.4]	0.0	0.0	0.7	0.0	0.7393
Chloramphenicol	4.6 [3.1-6.5]	1.2	3.7	6.1	0.0	0.0450
Florfenicol	0.5 [0.1-1.4]	0.0	3.7	0.5	0.0	0.0812
Gentamicin	7.6 [5.7-9.9]	11.6	0.0	8.3	1.4	0.0358
Neomycin	14.9 [12.2-17.9]	5.8	7.4	19.1	2.7	<0.0001
Tobramycin	7.1 [5.2-9.4]	9.3	0.0	8.1	1.4	0.0735
Amikacin	0.3 [0.0-1.1]	2.3	0.0	0.0	0.0	0.0052
Kanamycin	18.7 [15.7-21.9]	12.8	3.7	23.3	2.7	<0.0001

Streptomycin	20.6 [17.5-23.9]	8.1	7.4	26.7	2.7	<0.0001
Erythromycin	20.1 [17.0-23.4]	25.6	11.1	22.2	4.1	0.0012
Clindamycin	17.1 [14.2-20.3]	12.8	7.4	20.6	4.1	0.0014
Fusidic acid	4.1 [2.7-6.0]	24.4	3.7	0.9	0.0	<0.0001
Mupirocin	0.2 [0.0-0.9]	0.0	0.0	0.0	1.4	0.0534
Sulfamethoxazole/Trimethoprim	13.3 [10.7-16.2]	8.1	0.0	16.4	5.5	0.0034
Sulphonamides	46.8 [43.0-50.7]	29.1	11.1	54.3	35.6	<0.0001
Trimethoprim	16.9 [14.1-20.1]	12.8	3.7	20.0	8.2	0.0105
Rifampicin	1.9 [1.0-3.3]	3.5	0.0	1.4	4.1	0.2272
Resistance to at least 1 AB	79.4 [76.1-82.5]	74.4	81.5	82.7	64.4	0.0023
Resistance to ≥ 3 AB	35.0 [31.3-38.8]	34.9	25.9	39.0	13.7	0.0003

[a]Used for *S. aureus* and coagulase-negative staphylococci.
[b]Used for *S. pseudintermedius*.
All strains were susceptible to netilmicin, vancomycin, teicoplanin, linezolid and quinupristin/dalfopristin.

Table 3. Frequency of resistance genes for the total isolates and per staphylococcal strain. The p-value refers to the association between antimicrobial resistance genes and staphylococcal species.

Resistance gene	Percentage of the resistance gene in all strains (CI)	Percentage of the resistance gene in CoNS	Percentage of the resistance gene in *S. aureus*	Percentage of the resistance gene in *S. pseudintermedius*	Percentage of the resistance gene in *S. schleiferi*	p-value
mecA	11.6 [9.2-14.3]	26.7	40.7	8.7	0.0	<0.0001
blaZ	59.0 [55.1-62.9]	40.7	77.8	64.6	39.7	<0.0001
erm(A)	0.8 [0.3-1.8]	4.7	3.7	0.0	0.0	<0.0001
erm(B)	18.2 [15.3-21.4]	12.8	3.7	22.4	4.1	0.0001
erm(C)	2.4 [1.3-3.9]	11.6	7.4	0.2	2.7	<0.0001
cat pC221	4.1 [2.7-6.0]	1.2	0.0	5.6	0.0	0.0335
aphA3	18.0 [15.1-21.3]	7.0	11.1	23.3	1.4	<0.0001
aacA-aphD	7.6 [5.7-9.9]	11.6	3.7	8.1	1.4	0.0803
aadD	0.5 [0.0-1.0]	3.5	0.0	0.0	0.0	0.0003
aadE	16.3 [13.5-19.4]	0.0	0.0	23.1	0.0	<0.0001
tet(K)	9.3 [7.2-11.9]	20.9	0.0	9.2	0.0	<0.0001
tet(M)	27.5 [24.1-31.2]	4.7	3.7	37.2	4.1	<0.0001
dfr(K)	0.2 [0.0-0.9]	0.0	0.0	0.2	0.0	0.9366
dfr(G)	7.0 [5.1-9.2]	3.5	3.7	9.0	0.0	0.0163
msrA	1.9 [1.0-3.3]	9.3	3.7	0.5	1.4	<0.0001
mph(C)	2.2 [1.2-3.7]	10.5	3.7	0.7	1.4	<0.0001
fusB	2.1 [1.1-3.5]	12.8	0.0	0.2	1.4	<0.0001
fusC	0.8 [0.3-1.8]	4.7	3.7	0.0	0.0	<0.0001

Abbreviations: CoNS, coagulase negative staphylococci.

The variables gender and age were not considered risk factors ($p>0.05$) leading to more antimicrobial resistance. However, isolates from dogs were resistant to more antimicrobials (Table 4) then isolates from cats ($p<0.05$). Likewise, staphylococcal isolates from otitis were resistant to more antimicrobials than isolates from pyoderma or UTI ($p<0.05$, Table 4). No significant differences were found between isolates from pyoderma and isolates from UTI ($p>0.05$).

Using a logistic regression analysis, we assessed the trends over time in resistance to the different antimicrobials. Among the 38 antimicrobials analysed, resistance increased over the period analysed ($p<0.05$) in 27 antimicrobials (Figure 1), and the number of isolates with resistance to at least one antimicrobial or with multiple drug resistance also increased over time ($P<0.05$, Figure 1). The number of *mecA* positive isolates also increased over time ($p<0.0001$, Figure 2). The antimicrobials where resistance did not increase significantly ($p>0.05$) over the 16-year period under analysis were amikacin, florfenicol, fusidic acid, mupirocin, nitrofurantoin and rifampicin. The corresponding OR, CI and p-values are present in Supplementary Table 1.

The characteristics of all MRS isolates are shown in Supplementary Table 2. Eighteen *spa* types were identified in *S. aureus* (t002, t025, t032, t044, t084, t085, t091, t105, t108, t148, t311, t1294, t1346, t1897, t2357, t11188), including two new *spa* types: t14112 and t14113. The MRSA isolates were divided into 3 PFGE clusters (Supplementary Figure 2) and MLST included ST22-IV (n=8, including t025, t032 and t2357), ST105-II (n=1, t002), ST398-V (n=1, t108) and ST5-VI (n=1, t311). Isolates ST5 and ST105 belonged to clonal complex (CC) 5.

Most MRSE isolates were members of CC 5: ST2-nt (n=2), ST5-nt (n=2), ST20-nt (n=1), ST23-IV (n=1), ST35-nt (n=1), ST57-IV (n=1), ST190-nt (n=1) and a new ST (n=1), which carried an SCC*mec* II. The MRSE isolates were divided into 8 PFGE clusters (Supplementary Figure 2), with the two MRSE ST2 isolates having <80% similarity by PFGE.

MRSP were grouped into 5 PFGE clusters (Supplementary Figure 2) and included ST45 (n=1), ST71 (n=13), ST195 (n=1), ST196 (n=1), ST203 (n=1), ST339 (n=1), ST342 (n=2) and the new ST, assigned ST400 (n=3). The ST71, ST195 and ST203 belonged to CC71, while ST342 belonged to CC261, ST45 to CC45, ST196 to CC196 and ST339 to CC84. The isolates for which the MLST was not performed, grouped in the same PFGE cluster and so we assumed they belonged to CC71. The MRSP ST45 strain was non-typeable by SCC*mec* typing and by PFGE *Sma*I-macrorestriction.

Methicillin-resistant *S. haemolyticus* (MRSH) were divided into two PFGE clusters (Supplementary Figure 2) and SCC*mec* V (n=5) was the most frequent type, followed by nt (n=3).

Table 4. Risk factors and corresponding OR, CI and p-value for the occurrence of individual antimicrobial resistance and *mecA* gene, from logistic regression.

Risk factor	Antimicrobial/gene	Odds Ratio	Confidence Interval	p-value
Dogs[a]				
	OXA	6.4	2.2-18.4	0.0006
	ENR	2.8	1.6-4.9	0.0005
	CIP	2.8	1.6-4.9	0.0005
	LEV	3.1	1.7-5.6	0.0001
	NOR	3.1	1.8-5.5	<0.0001
	OFX	3.1	1.8-5.4	<0.0001
	MXF	3.1	1.7-5.7	0.0002
	TET	0.3	0.2-0.6	0.0005
	GEN	2.8	1.4-5.5	0.0036
	TOB	3.0	1.5-6.0	0.0017
	FUS	3.9	1.6-9.6	0.0026
	mecA	3.1	1.7-5.4	0.0002
Otitis[b]				
	FOX	3.7	1.2-11.1	0.0191
	OXA	4.8	2.0-11.7	0.0006
	ENR	2.3	1.3-4.1	0.0051
	CIP	2.3	1.3-4.1	0.0051
	LEV	2.6	1.4-4.7	0.0024
	NOR	2.1	1.2-3.7	0.0109
	OFX	2.0	1.1-3.6	0.0154
	MXF	2.8	1.5-5.5	0.0020
	mecA	4.8	2.5-9.2	<0.0001
Otitis[c]				
	ENR	2.3	1.1-4.6	0.0241
	CIP	2.3	1.1-4.6	0.0241
	LEV	2.4	1.1-5.1	0.0200
	NOR	2.1	1.0-4.2	0.0421
	OFX	2.0	1.0-4.0	0.0543
	MXF	2.8	1.3-6.1	0.0109
	KAN	0.5	0.2-0.9	0.0458
	FUS	4.2	1.4-12.9	0.0116
	mecA	2.9	1.3-6.6	0.0116

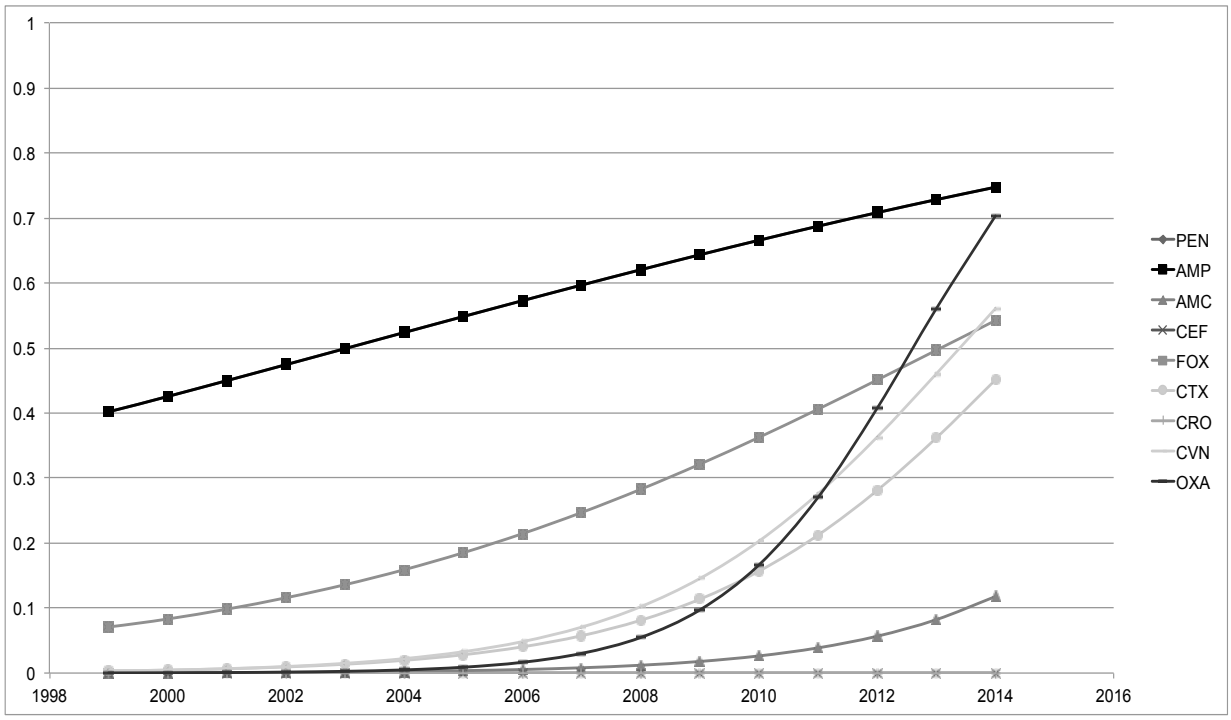
[a]OR relative to isolates from cats.

[b]OR relative to isolates from pyoderma.

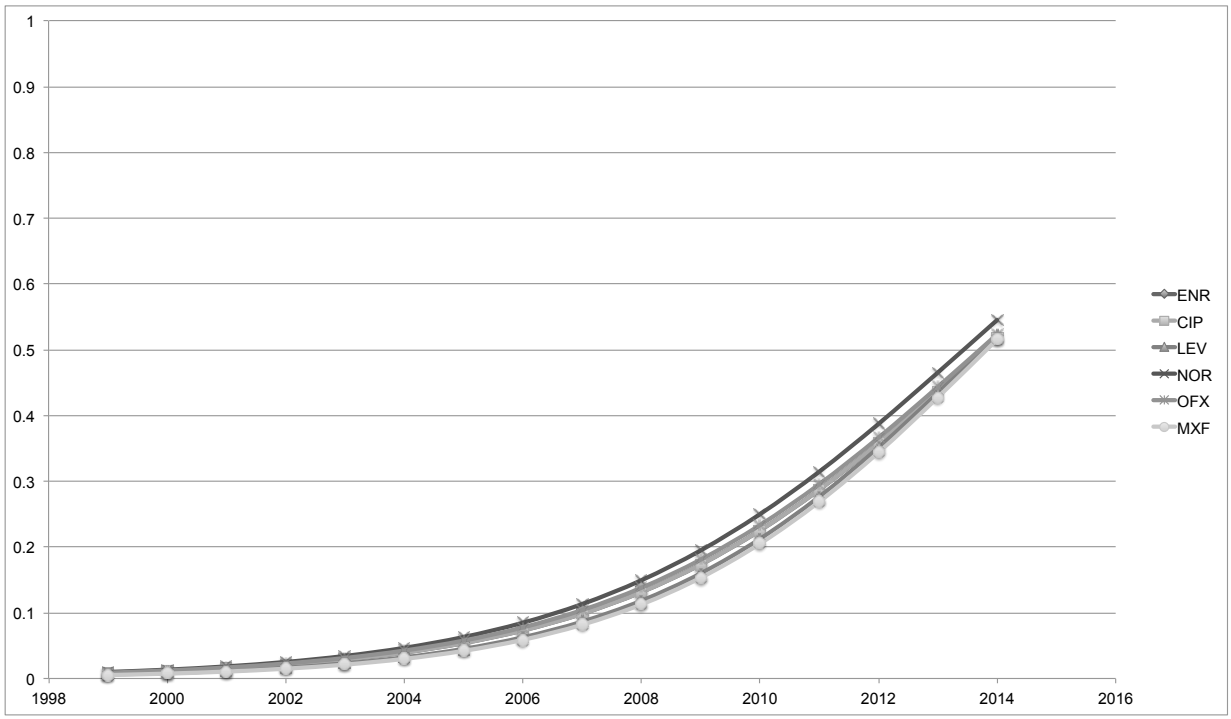
[c]OR relative to isolates from UTI.

Figures 1. Significant evolution of antimicrobial resistance over the 16 years studied ($p<0.05$): a) β-lactams; b) Fluoroquinolones; c) Aminoglycosides; d) Folate pathway inhibitors; e) Other antimicrobials.

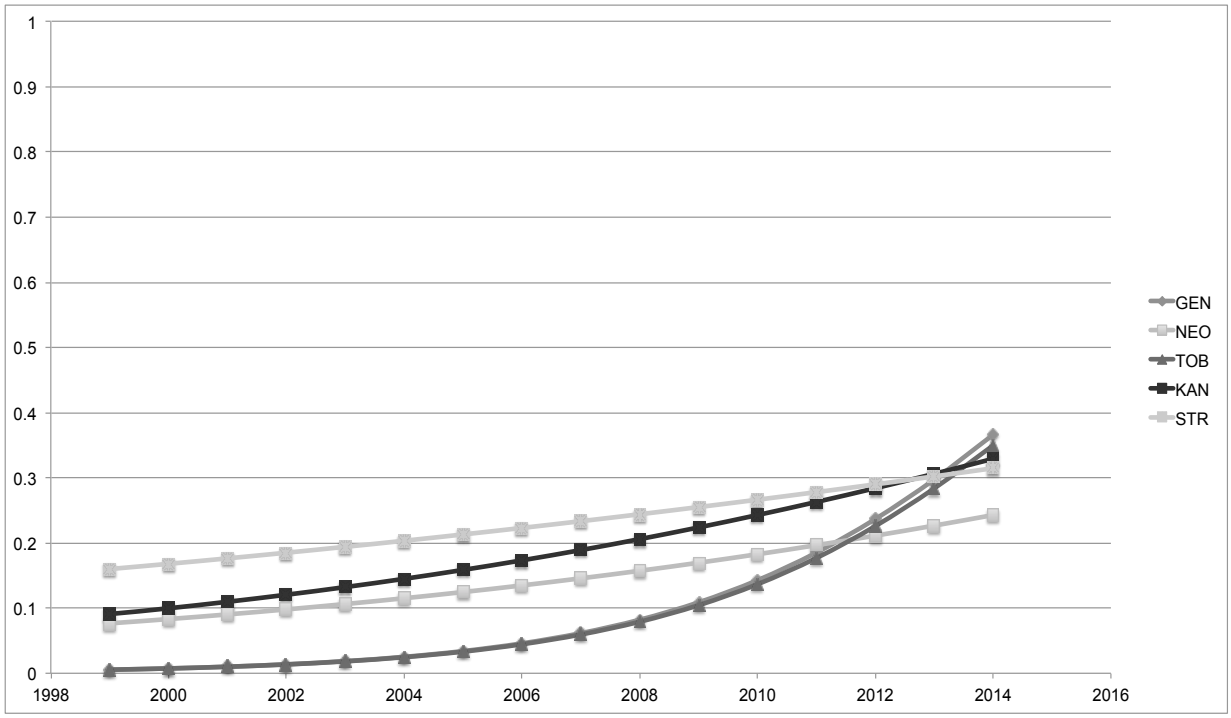
a) β-lactams



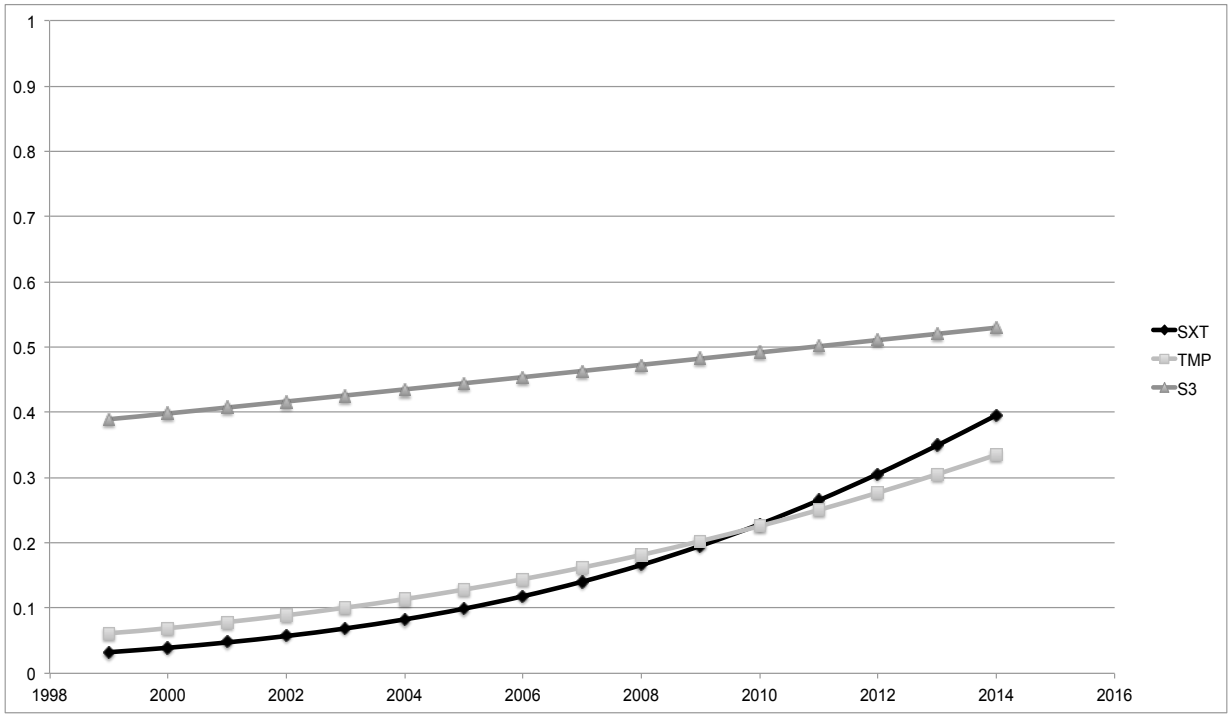
b) Fluoroquinolones



c) Aminoglycosides



d) Folate pathway inhibitors



e) Other antimicrobials

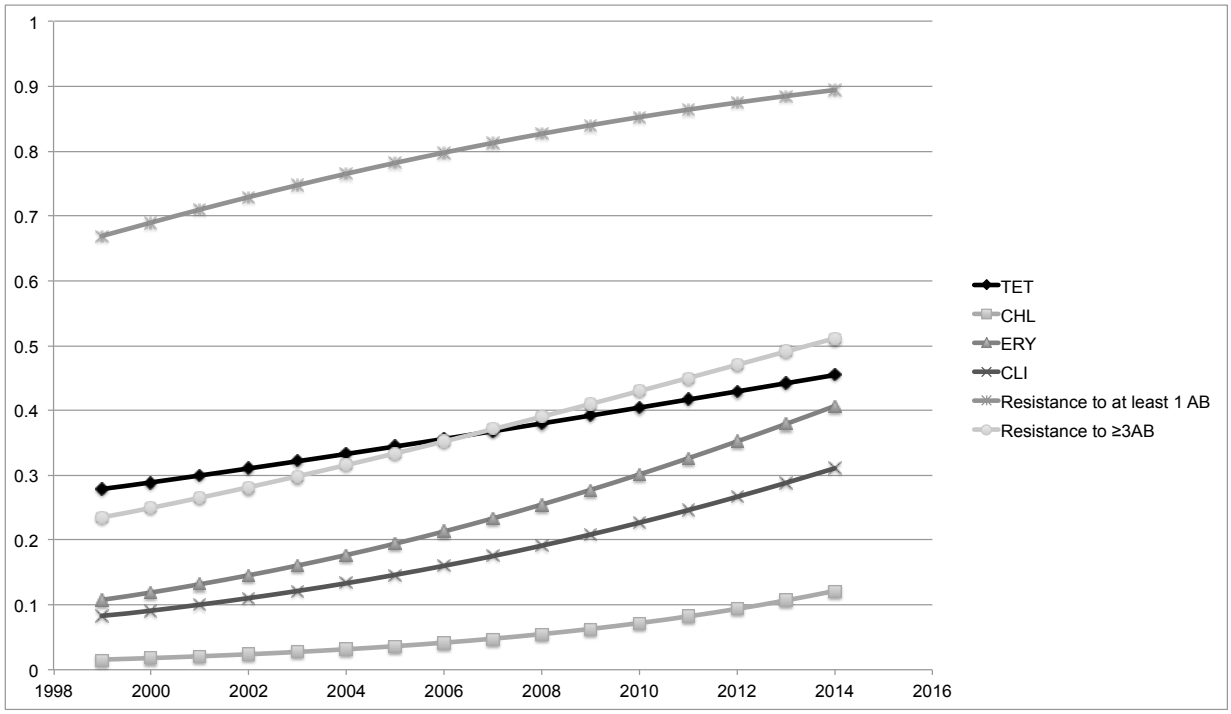
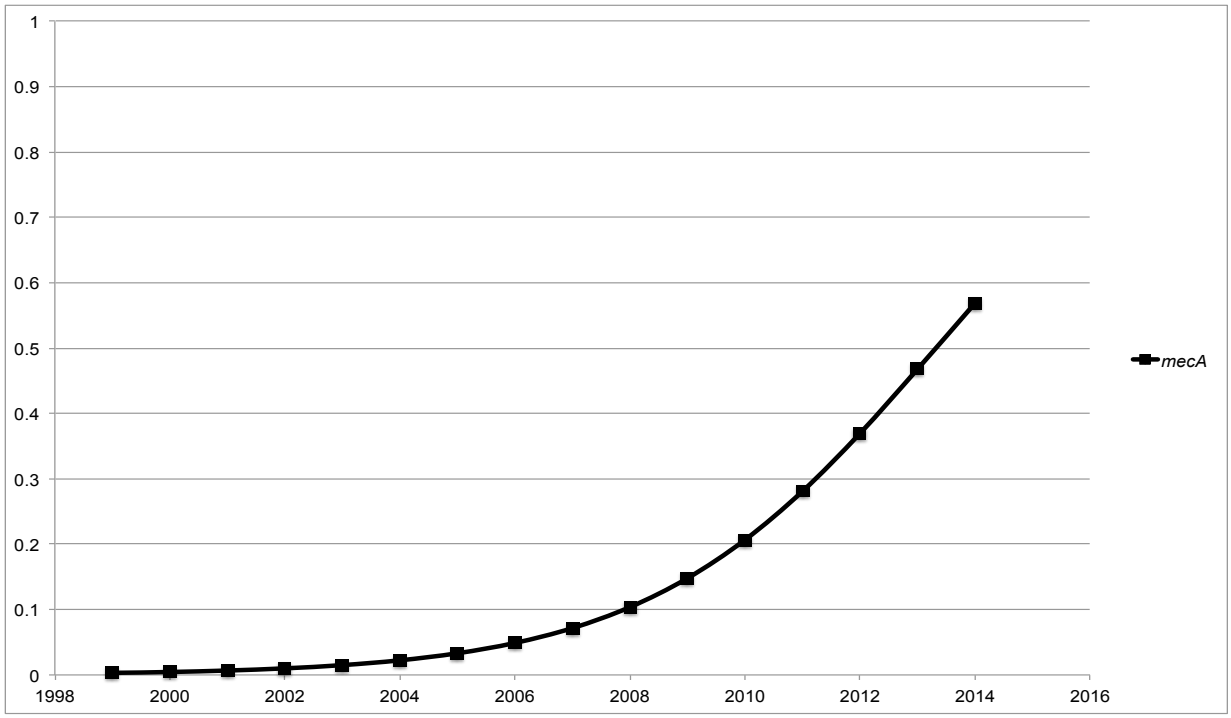


Figure 2. Significant evolution of *mecA*-positive strains over the 16 years studied ($p<0.05$).



Discussion

Recent studies have evaluated antimicrobial resistance in *S. pseudintermedius*.[2,24,25] However, none of these studies combined all staphylococcal species found in clinical specimens, susceptibility for several antimicrobials, corresponding resistance mechanisms and, most importantly, molecular epidemiology of MRS. Moreover, we studied a large period of time, 16 years, to establish trends in antimicrobial resistance and changes in genotypes.

The overall prevalence of clinical MRS isolates found in this study was 11.7%, which is higher than the prevalence found in a similar study made in Lithuania (5.3%).[26] This difference could be due to a higher consumption of first and second generation cephalosporins in Portugal compared to Lithuania.[27] However, the prevalence of MRSA within *S. aureus* isolates (40.7%) and MRSP within *S. pseudintermedius* (8.7%) was similar to two studies made in Germany (ranging from 41.3% to 62.7% MRSA in *S. aureus* of canine, feline and equine origin; and 6.3% of MRSP within isolates belonging to the *S. intermedius* group).[28,29] In Italy, a much higher prevalence of MRSP was found in clinical samples (21% of MRSP within isolates belonging to the *S. intermedius* group).[30] Interestingly, Germany and Portugal have similar consumptions of first and second generation cephalosporins, but in Italy it is higher.[27] We could speculate that cephalosporins may select for methicillin-resistant isolates, but further studies are needed.

Most of our isolates were characterized as *S. pseudintermedius*, which was expected since most isolates were from dogs, where this species is the most frequently found.[1] Recently, two studies evaluated antimicrobial resistance in *S. pseudintermedius* over time and detected trends of increasing resistance for ampicillin/amoxicillin/penicillin, cefovecin, cephalexin, enrofloxacin, clindamycin and sulfamethoxazole/trimethoprim.[24,25] In our study, trends of increasing resistance to these antimicrobials were also observed, but we detected other trends of increasing resistance, including cefoxitin in *S. aureus* and CoNS, oxacillin in *S. pseudintermedius*, ciprofloxacin, norfloxacin, ofloxacin, moxifloxacin, tetracycline, chloramphenicol, gentamicin, neomycin, tobramycin, kanamycin, streptomycin, erythromycin, sulphonamides and trimethoprim in all staphylococcal groups analysed. Moreover, increasing trends of resistance to at least 1 antimicrobial and multidrug-resistance were also identified, such that almost 35% of the staphylococcal isolates were multidrug-resistant. The most common multidrug-resistant pattern among the methicillin-susceptible isolates was ampicillin/penicillin-tetracycline-sulphonamides and in methicillin-resistant isolates it was ß-lactams-fluoroquinolones-tetracycline. These resistance profiles are in accordance with the antimicrobial usage patterns in companion animal practice in Portugal.[27] In fact, penicillins+beta-lactamase inhibitors, 1st and 2nd generation cephalosporins, fluoroquinolones and tetracyclines are among the antimicrobials most used (in this order)

by companion animal practitioners in this country.[27]

Isolates from otitis were more resistant to several antimicrobials and carried more often the *mecA* gene than isolates from pyoderma or UTI. This finding suggests that these antimicrobials are probably being used inappropriately for the treatment of otitis. The recommended treatment option for otitis externa is antiseptics however, in some cases (e.g. ulceration and/or tympanic membrane rupture) there is a need for the administration of systemic antimicrobial therapy, and the first-line antimicrobials are ß-lactams or fluoroquinolones.[31-33] However, antimicrobials that are used systemically for otitis are unlikely to achieve therapeutic concentrations within the fluid and waxy exudates of the external canals in which the infectious organisms are harboured. [26] Our study, probably, reflects the selective pressure imposed on staphylococci in the ear by the use of these antimicrobials, including the higher frequency of *mecA*-positive staphylococci, further supporting the urgent need for more studies on the efficacy of systemic antimicrobials on ear infections.

There are no cefoxitin or oxacillin recommended breakpoints for *S. schleiferi* subsp. *coagulans*. However, these breakpoints are important for diagnostic purposes, since this species is very common in companion animals. Although we did not find *mecA*-positive isolates, there are already descriptions of methicillin-resistant *S. schleiferi* subsp. *coagulans* isolates[27] and so it is urgent to determine appropriate breakpoints for this species. Moreover, with the increasing frequency of multidrug-resistant strains, some antimicrobials are being suggested as second-line antimicrobial agents, namely florfenicol, amikacin, minocycline, doxycycline, nitrofurantoin, topical fusidic acid or mupirocin.[34-36] Even some antimicrobials that are used daily in companion animal practice, like ampicillin, cefovecin or sulfamethoxazole/trimethoprim do not have clinical breakpoints determined for these species. Thus, updated and species-specific clinical breakpoints are essential for the appropriate selection of antimicrobials.

Interestingly, the first *mecA*-positive isolate detected in our study was an MRSA isolated in 2001. The ST22-IV, which represents the EMRSA-15, was the most common MRSA lineage found in this study. This is in agreement with previous reports, which show that there is a shared population of this lineage infecting/colonizing humans and companion animals.[37] The ST22-t032-SCC*mec* IV isolates were negative for the *erm*(C), while the other ST22-IV non-t032 (t2357 and t025) isolates carried the *erm*(C) gene. It is assumed that the loss of this gene is associated with isolates coming from companion animals,[37] which suggests that the MRSA ST22 non-t032 isolates found in our study were acquired from humans very recently, and they have thus maintained the *erm*(C) gene.

Only one MRSA strain was ST398-t108-V, a livestock-associated MRSA, and it was isolated from a dog. This strain had 93% ApaI-PFGE similarity to previously isolated MRSA isolates from calves in Portugal (data not shown).[38] Surprisingly, all these isolates

(from dog and calves) carried the *fexA* gene and were resistant to fluoroquinolones, which suggests that they had a similar source of infection/colonization.[38] However, the dog in our study had no history of contact with farms or farm animals, so this remains to be elucidated. The MRSA ST5-t311-VI isolated from a cat was resistant to FUS and carried the *fusC* gene, as was reported for one MRSA ST5-t062-VI isolated from a horse in a previous study.[22] Comparing the SmaI-PFGE profiles of these two isolates (data not shown) they had 86% similarity. As the cat's strain was isolated in 2001 we could not determine if there was any history of contact with horses and so we could not define the source of infection/colonization. However, it is interesting to notice that several MRSA lineages are disseminated in different animal species.

The first MRSP strain was identified in Portugal in 2007, but only in 2010 we detected an increase in the number of isolates. The first MRSP isolates in Europe were detected in 2005,[39] and were ST71-II-III. Interestingly, the first MRSP strain in Portugal, isolated in 2007, was ST196-V. Only in 2009 the first ST71-II-III appeared in Portugal. Between 2009 and mid-2012, MRSP CC71-II-III was the only lineage detected. Yet, in 2013-2014 we observed a higher genetic diversity among the MRSP isolates isolated, with other MRSP lineages appearing, including a new ST (ST400) carrying the *mecA* gene. The ST45-nt, ST339-III and ST342-IV lineages were already described in recent studies.[2,40] The ST45 was the predominant MRSP clonal lineage in Thailand and Israel, and was not typeable by SmaI-PFGE and SCC*mec* typing.[40] This lineage carried a novel pseudo-SCC*mec* element, ΨSCC-*mec*$_{57395}$ that, besides *mecA*, also carried determinants of resistance to heavy metals, such as arsenic, cadmium, and copper.[40] It seems that this ST has also been introduced in Europe, as the MLST database reports that ST45 has been detected in England, The Netherlands and now in Portugal. The new ST, ST400, does not belong to any of the previous *mecA*-positive clonal complexes, which suggests that SCC*mec* has been acquired by this ST. Two of the MRSP ST400 isolates were isolated from two dogs that lived in the same kennel. However, the third dog had no connection to these dogs or to the kennel, which could mean that this lineage is already spreading through the dogs' population in Portugal.

The *fexA* gene was detected in 3 isolates (2 *S. pseudintermedius* and 1 *S. aureus*). The animals (three dogs) infected with these isolates had been previously diagnosed with an infection caused by a multidrug-resistant MRSA or MRSP strain and so florfenicol (25-50mg/kg q12h SC, Nuflor®, Merck Animal Health, USA) was being used as a last-resort antimicrobial. The use of florfenicol was very recently suggested, as a second-line antimicrobial agent in dogs.[34] However, it seems that the use of this antimicrobial can lead to additional acquisition of antimicrobial resistance genes or isolates. Furthermore, one of these isolates (an *S. pseudintermedius*), also carried the *cfr* gene and, to the best of our knowledge, this is the first description the *cfr* gene in a *S.*

pseudintermedius strain isolated from a dog under flofenicol treatment. Although the strain did not exhibit resistance to linezolid, this is a worrisome finding, since it shows *S. pseudintermedius* could be carriers of important resistance genes.

The MRSE STs found in this study were identical to the ones isolated in humans in Portugal (community- and hospital-acquired isolates).[41] This means that MRSE isolates can circulate between humans and animals, making these a reservoir of important MRSE lineages. Unfortunately, there is no MLST database for *S. haemolyticus*, which makes it impossible to compare our isolates to other animal or even human isolates. Either way, it is important to notice that MRCoNS were more frequently isolated than could be expected from previous studies, and the presence of the *mecA* gene was highly associated with these isolates. Furthermore, several MRCoNS exhibited a multidrug-resistance pattern, suggesting that they are reservoirs of antimicrobial resistance genes.

The results here reported might be a biased representation of the reality found in companion animals in Portugal, as they were obtained from a reference laboratory that receives samples from complicated infections observed in private practices. This suggests that our results only represent a small part of the staphylococci isolates that in reality infect companion animals. However, since our laboratory has been collecting samples since 1999, the increased frequency of antimicrobial resistance over time reported here probably reflects what is happening in the staphylococci population in general. Additionally, the observed time-trend for the various antimicrobials reflects the development of new resistant strains but also the spread of resistant organisms over time.

This study highlights the importance of companion animals as reservoirs of important antimicrobial-resistant pathogens. In 2005, Heuer and colleagues underlined that the use of antimicrobial drugs in companion animals had received little attention and that monitoring programs had focused solely on antimicrobial drug consumption in food animals.[42] Ten years later, the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) group reports information on the sales of tablets by veterinary antimicrobial class for companion animals.[27] Yet, no alteration or restrictions in antimicrobial prescription in companion animals have been imposed so far (especially considering critically important antimicrobials). International and national guidelines on antimicrobial use for companion animal practice are urgently needed and in fact they have been published more frequently. Another problem that requires immediate attention is updated and species-specific clinical breakpoints. Together these features will hopefully improve antimicrobial stewardship and prevent the development of antimicrobial resistance.

The significant increase of antimicrobial-resistant and *mecA*-positive isolates in the last years is worrying. Furthermore, several isolates are multidrug-resistant, which complicates antimicrobial treatment and raises

the risk of transfer to humans or human isolates. Several clonal lineages of MRSA and MRSE circulating in human hospitals and in the community were found in this study, suggesting that companion animals can become infected with and contribute to the dissemination of highly successful human clones. Thus, companion animals can act as reservoirs of important human clones, perpetuating the transmission cycle of MRS between humans and companion animals.

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None to declare.

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Supplementary Table 1. Odds ratio, confidence intervals and p-value for the evolution over time of the proportion of isolates resistant to different antimicrobials and *mecA*.

Antimicrobial	Odds ratio	Confidence Interval	p-value
Ampicillin	1.104	1.060-1.150	<0.001
Penicillin	1.104	1.060-1.150	<0.001
Amoxicillin/clavulanic acid	1.487	1.341-1.648	<0.001
Oxacillin	1.851	1.558-2.199	<0.001
Cefoxitin	1.202	1.080-1.377	<0.001
Cefovecin	1.491	1.362-1.632	<0.001
Cefotaxime	1.444	1.315-1.585	<0.001
Ceftriaxone	1.419	1.296-1.552	<0.001
Cephalexin	1.607	1.401-1.843	<0.001
Enrofloxacin	1.382	1.284-1.486	<0.001
Ciprofloxacin	1.382	1.284-1.486	<0.001
Levofloxacin	1.413	1.306-1.528	<0.001
Norfloxacin	1.373	1.278-1.475	<0.001
Ofloxacin	1.374	1.280-1.476	<0.001
Moxifloxacin	1.420	1.306-1.544	<0.001
Gentamicin	1.357	1.243-1.482	<0.001
Neomycin	1.090	1.034-1.149	0.001
Tobramycin	1.350	1.234-1.476	<0.001
Kanamycin	1.109	1.056-1.164	<0.001
Streptomycin	1.057	1.009-1.106	0.020
Sulfamethoxazole/trimethoprim	1.218	1.149-1.293	<0.001
Sulphonamides	1.037	0.999-1.078	0.058
Trimethoprim	1.145	1.088-1.205	<0.001
Tetracycline	1.050	1.009-1.092	0.016
Chloramphenicol	1.151	1.052-1.259	0.002
Erythromycin	1.120	1.068-1.174	<0.001
Clindamycin	1.110	1.056-1.167	<0.001
Resistance to at least 1 AB	1.101	1.047-1.159	<0.001
Resistance to ≥ 3 AB	1.083	1.040-1.127	<0.001
mecA	1.496	1.371-1.631	<0.001

Supplementary Table 2. Characteristics of the methicillin-resistant staphylococci found in this study.

Isolate	Source	Origin	Staphylococcal species	SCC*mec* type	*spa* type	ST	CC	Co-resistance	Co-resistance genes
FMV1873/01	Urinary tract infection	Cat	*S. aureus*	VI	t311	5	5	NEO, STR, FUS, SUL	*blaZ, mecA, fusC*
FMV52/02	Urinary tract infection	Cat	*S. epidermidis*	nt	-	20	5	FQ, STR, ERY, RIF	*blaZ, mecA, msrA*
FMV850B/02	Otitis	Cat	*S. epidermidis*	nt	-	2	5	CIP, ENR, NOR, OFX, LEV, GEN, NEO, TOB, AMK, KAN, STR, SXT, TMP, SUL	*blaZ, mecA, aacA-aphD, aadD*
FMV2985/02	Otitis	Dog	*S. haemolyticus*	V	-	-	-	FQ, TET, GEN, KAN, ERY	*blaZ, erm*(C)*, tet*(K)*, aacA-aphD, aphA3*
FMV3496/02	Urinary tract infection	Cat	*S. epidermidis*	IV	-	23	5	FQ, GEN, NEO, TOB, AMK, KAN, STR, ERY, CLI, FUS, RIF	*blaZ. mecA, erm*(A)*, aacA-aphD, aadD, fusB*
FMV3951/04	Otitis	Dog	*S. haemolyticus*	nt	-	-	-	NEO, KAN	*blaZ, mecA, aphA3*
FMV1535/06	Pyoderma	Dog	*S. epidermidis*	nt	-	190	5	TET, STR	*blaZ, mecA, tet*(K)
FMV2995/06	Urinary tract infection	Cat	*S. lentus*	III	-	-	-	FQ, TET, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(A)*, erm*(C)*, tet*(M)*, tet*(K)*, mph*(C)
FMV6133/06	Urinary tract infection	Cat	*S. epidermidis*	nt	-	2	5	GEN, TOB, KAN	*blaZ, mecA, aacA-aphD, aphA3*
FMV1879B/07	Urinary tract infection	Cat	*S. pseudintermedius*	V	-	196	196	FQ, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV5628/07	Otitis	Dog	*S. haemolyticus*	V	-	-	-	FQ, GEN, NEO, KAN, ERY,	*blaZ, mecA, aacA-aphD,*

								FUS, SXT, TMP, SUL	*aphA3, msrA, mph*(C), *fusC, dfr*(G)
FMV1504A/08	Pyoderma	Dog	*S. aureus*	IV	t032	22	22	FQ	*blaZ, mecA*
FMV4331/08	Urinary tract infection	Cat	*S. epidermidis*	nt	-	35	5	FQ, STR, ERY, FUS, SUL	*blaZ, mecA, erm*(B)*, fusB*
FMV3021/09	Otitis	Cat	*S. haemolyticus*	nt	-	-	-	FQ, GEN, NEO, TOB, KAN, FUS, SXT, TMP, SUL	*blaZ, mecA, aacA-aphD, aphA3, fusC, dfr*(G)
FMV3891/09	Urinary tract infection	Cat	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV1860/10	Otitis	Cat	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, GEN, N, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV3008/10	Otitis	Dog	*S. pseudintermedius*	II-III	-	203	71	FQ, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV3607/10	Urinary tract infection	Cat	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, CHL, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, cat* pC221*, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV4877/10	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV5819/10	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL, RIF	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)*,*

FMV6096B/10	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV981/11	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, GEN, N, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV7/2011	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, CHL, FFC, GEN, NEO, TOB, KAN, STR, ERY, CLI, FUS, SXT, TMP, SUL, RIF	*blaZ, mecA, fexA, erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, fusB, dfr*(G)
FMV9/2011	Pyoderma	Dog	*S. haemolyticus*	V	-	-	-	FQ, TET, ERY, FUS	*blaZ, mecA, erm*(B), *erm*(C), *tet*(M), *tet*(K), *fusB*
FMV13/2011	Discospon-dylitis	Dog	*S. pseudintermedius*	II-III	-	195	71	FQ, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *aacA-aphD, aphA3, aa-dE, dfr*(G)
FMV15/2011	Surgical site infection	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV16/2011	Surgical site infection	Dog	*S. pseudintermedius*	nt	-	nd	71	FQ, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *aacA-aphD, aphA3, aa-dE, dfr*(G)
FMV17/2011	Surgical site infection	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, N, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV24/2011	Synovitis	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, NEO, TOB,	*blaZ, mecA, erm*(B),

								KAN, STR, ERY, CLI, SXT, TMP, SUL	*tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV4465/12	Pyoderma	Dog	*S. aureus*	IV	t032	22	22	FQ	*blaZ, mecA*
FMV1/2012	Wound	Cat	*S. aureus*	IV	t032	22	22	FQ	*blaZ, mecA*
FMV2/2012	Wound	Cat	*S. aureus*	IV	t032	22	22	FQ	*blaZ, mecA*
FMV14/2012	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, NEO, TOB, KAN, STR, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *aphA3, aadE, dfr*(G)
FMV21/2012	Pyoderma	Dog	*S. haemolyticus*	nt	-	-	-	FQ, TET, ERY, FUS	*blaZ, mecA, erm*(B), *erm*(C), *tet*(M), *fusB*
FMV23/2012	Wound	Cat	*S. aureus*	IV	t032	22	22	FQ	*blaZ, mecA*
FMV29/2012	Urinary tract infection	Cat	*S. pseudintermedius*	II-III	-	71	71	FQ, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV34/2012	Pyoderma	Dog	*S. pseudintermedius*	nt	-	45	45	FQ, TET, CHL, GEN, N, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, cat* pC221, *erm*(B), *tet*(M), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV41/2012	Rhinitis	Cat	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV47/2012	Otitis	Dog	*S. epidermidis*	nt	-	5	5	TET, SXT, TMP, SUL	*blaZ, mecA*
FMV48/2012	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV52/2012	Surgical site infection	Dog	*S. epidermidis*	II	-			ERY	*blaZ, mecA, mph*(C), *msrA*

FMV54/2012	Synovitis	Dog	*S. pseudintermedius*	nt	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV58/2012	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm(B),* *tet*(K), *aacA-aphD, aphA3, aadE, dfr(G)*
FMV59/2012	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV60/2012	Pyoderma	Dog	*S. epidermidis*	IV	-	5	5	CIP, ENR, LEV, NOR, OFX, TET, GEN, TOB, KAN, ERY, CLI, FUS, SUL	*blaZ, mecA, erm*(C), *tet*(K), *aacA-aphD, fusB*
FMV61/2012	Surgical site infection	Dog	*S. epidermidis*	IV	-	57	5	SUL	*mecA*
FMV62/2012	Otitis	Cat	*S. epidermidis*	nt	-	5	5	NOR, OFX, TET, GEN, TOB, KAN, ERY, CLI	*blaZ, mecA, erm*(C), *tet*(K), *aacA-aphD,*
FMV64/2012	Pyoderma	Dog	*S. pseudintermedius*	nt	-	nd	71	FQ, TET, CHL, GEN, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, cat* pC221, *erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV71/2012	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B), *tet*(K), *aacA-aphD, aphA3, aadE, dfr*(G)
FMV72/2012	Urinary tract infection	Dog	*S. aureus*	II	t002	105	5	FQ, ERY, CLI	*blaZ, mecA, erm*(A)
FMV22/2013	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	71	71	FQ, TET, GEN, NEO, TOB,	*blaZ, mecA, erm*(B),

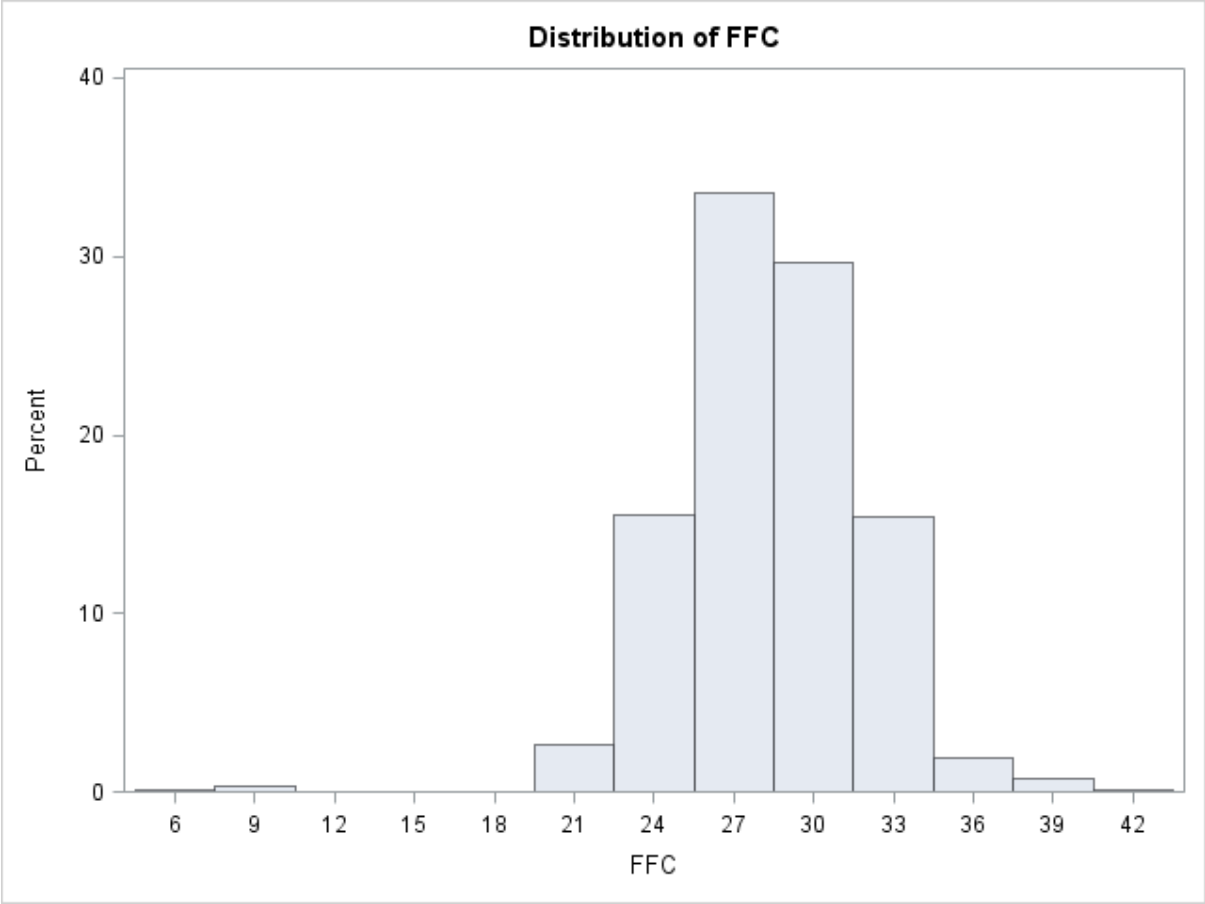
								KAN, STR, ERY, CLI, SXT, TMP, SUL	*tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV25/2013	Meningitis	Dog	*S. hominis*	nt	-	-	-	FUS, SUL	*blaZ, mecA, fusB*
FMV29/2013	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV30/2013	Pyoderma	Dog	*S. aureus*	V	t108	398	398	FQ, TET, CHL, FFC	*blaZ, mecA, tet*(M)*, fexA*
FMV37/2013	Pyoderma	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV52/2013	Pyoderma	Dog	*S. pseudintermedius*	IV	-	342	261	TET, NEO, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(M)*, aphA3, aadE, dfr*(G)
FMV53/2013A	Otitis (right ear)	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV53/2013B	Otitis (left ear)	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV56/2013A	Pyoderma	Dog	*S. pseudintermedius*	III	-	400	400	CIP, ENR, LEV, NOR, OFX, TET, CHL, NEO, KAN, STR, ERY, CLI	*blaZ, mecA, cat* pC221*, erm*(B)*, tet*(M)*, aphA3, aadE*
FMV56/2013B	Pyoderma	Dog	*S. haemolyticus*	V	-	-	-	TET, ERY, CLI, FUS	*blaZ, mecA, erm*(C)*, tet*(K)*, fusB*
FMV56/2013C	Pyoderma	Dog	*S. haemolyticus*	V	-	-	-	TET, SUL	*blaZ, mecA, tet*(K)
FMV57/2013A	Pyoderma	Dog	*S. pseudintermedius*	III	-	400	400	CIP, ENR, LEV, NOR, OFX,	*blaZ, mecA, cat* pC221*,*

								TET, CHL, NEO, KAN, STR, ERY, CLI	*erm*(B)*, tet*(M)*, aphA3, aadE*
FMV57/2013B	Pyoderma	Dog	*S. simulans*	III	-	-	-	TET	*blaZ, mecA, tet*(K)
FMV74/2013	Unknown	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV16/14	Surgical site infection	Dog	*S. pseudintermedius*	III	-	400	400	FQ, TET, CHL, NEO, KAN, STR, ERY, CLI	*blaZ, mecA, cat* pC221*, erm*(B)*, tet*(M)*, aphA3, aadE*
FMV9/2014	Pyoderma	Dog	*S. aureus*	IV	t2357	22	22	FQ, ERY, CLI	*blaZ, mecA, erm*(C)
FMV10/2014	Otitis	Dog	*S. pseudintermedius*	nt	-	71	71	CIP, ENR, LEV, NOR, OFX, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV12/2014	Pyoderma	Dog	*S. pseudintermedius*	IV	-	342	261	TET, N, KAN, STR, SXT, TMP, SUL	*blaZ, mecA, tet*(M)*, aphA3, aadE, dfr*(G)
FMV19/2014	Pyoderma	Dog	*S. aureus*	IV	t032	22	22	FQ	*blaZ, mecA*
FMV28/2014	Otitis	Dog	*S. pseudintermedius*	nt	-	nd	71	FQ, TET, GEN, NEO, TOB, KAN, STR, ERY, CLI, SXT, TMP, SUL	*blaZ, mecA, erm*(B)*, tet*(K)*, aacA-aphD, aphA3, aadE, dfr*(G)
FMV32/2014	Surgical site infection	Dog	*S. caprae*	nt	-	-	-	GEN, KAN, SUL	*blaZ, mecA, aacA-aphD*
FMV42/2014	Pyoderma	Dog	*S. pseudintermedius*	nt	-	339	84	FQ, NEO, KAN, STR, ERY, CLI, SXT, TMP, SUL, RIF	*blaZ, mecA, erm*(B)*, aphA3, dfr*(G)
FMV48/2014	Otitis	Dog	*S. pseudintermedius*	II-III	-	nd	71	FQ, GEN, TOB, KAN, STR, SXT, TMP, SUL	*blaZ, mecA, aacA-aphD, aphA3, aadE, dfr*(G)

FMV50/2014	Surgical site infection	Dog	*S. aureus*	IV	t025	22	22	FQ, ERY	*blaZ, mecA, erm*(C)

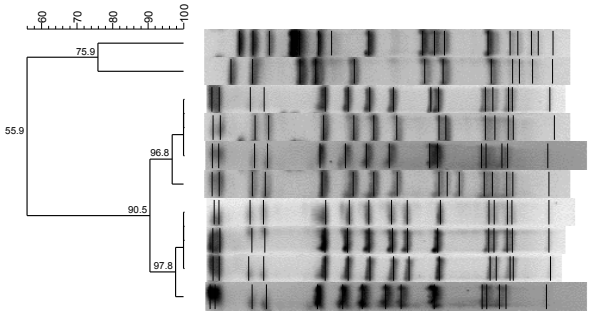
Abbreviations: AMK, amikacin; CHL, chloramphenicol; CIP, ciprofloxacin; CLI, clindamycin; ERY, erythromycin; FFC, florfenicol; FQ, fluoroquinolones; FUS, fusidic acid; GEN, gentamicin; KAN, kanamycin, MXF, moxifloxacin; NEO, neomycin; NOR, norfloxacin; OFX, ofloxacin; RIF, rifampicin; STR, streptomycin; SUL, sulphonamides; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; TOB, tobramycin.

Supplementary Figure 1. Distribution of the florfenicol zone diameters (in milimeters) identified in our study.



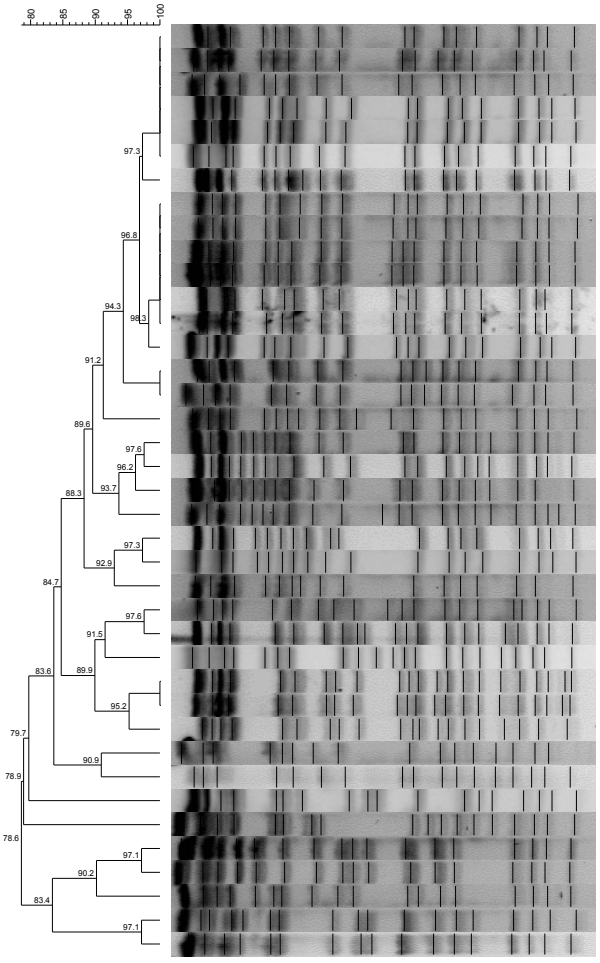
Supplementary Figure 2. Dendograms showing the similarity between (a) MRSA strains; (b) MRSP strains, (c) MRSE strains; and (d) MRSH strains. Clusters were defined as ≥ 80% similarity using Dice Coefficient (Tolerance 1.0%, Optimization 1.7%).

(a)



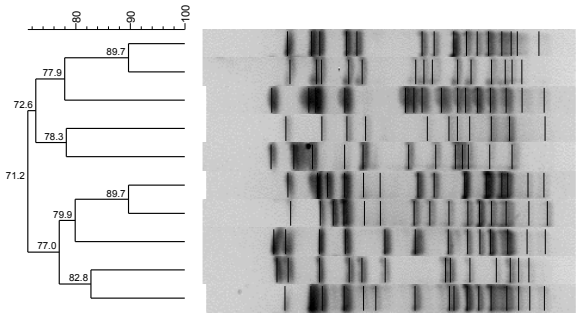
Cat	t311	ST5	Staphylococcus aureus	new
Dog	t002	ST105	Staphylococcus aureus	II
Cat	t032	ST22	Staphylococcus aureus	IV
Cat	t032	ST22	Staphylococcus aureus	IV
Dog	t2357	ST22	Staphylococcus aureus	IV
Dog	t025	ST22	Staphylococcus aureus	IV
Dog	t032	ST22	Staphylococcus aureus	IV
Cat	t032	ST22	Staphylococcus aureus	IV
Cat	t032	ST22	Staphylococcus aureus	IV
Dog	t032	ST22	Staphylococcus aureus	IV

(b)



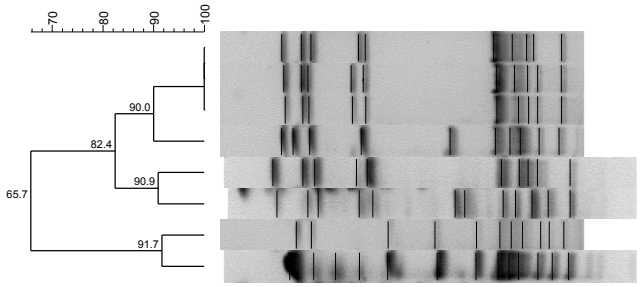
Synovitis	Dog	MRSP		II-III
UTI	Cat	MRSP	71	II-III
Pyoderma	Dog	MRSP		II-III
Otitis	Cat	MRSP	71	II-III
Otitis	Dog	MRSP	203	II-III
UTI	Cat	MRSP	71	II-III
	Dog	MRSP		II-III
Surgical site infec.	Dog	MRSP		II-III
Surgical site infec.	Dog	MRSP		II-III
Pyoderma	Dog	MRSP		II-III
Pyoderma	Dog	MRSP		II-III
Pyoderma	Dog	MRSP	71	II-III
Pyoderma	Dog	MRSP	71	II-III
Skin	Dog	MRSP	71	II-III
Pyoderma	Dog	MRSP		II-III
Otitis	Dog	MRSP		II-III
Pyoderma	Dog	MRSP	71	II-III
Pyoderma	Dog	MRSP	71	II-III
Rhinitis	Cat	MRSP		II-III
Pyoderma	Dog	MRSP	71	II-III
Pyoderma	Dog	MRSP		II-III
Pyoderma	Dog	MRSP		NT
UTI	Cat	MRSP	71	II-III
Synovitis	Dog	MRSP		NT
Surgical site infec.	Dog	MRSP		NT
Surgical site infec.	Dog	MRSP	195	II-III
Pyoderma	Dog	MRSP	71	II-III
Otitis	Dog	MRSP		II-III
Otitis	Dog	MRSP		II-III
Otitis	Dog	MRSP		NT
Otitis	Dog	MRSP	71	NT
Pyoderma	Dog	MRSP	71	II-III
Urine	Cat	MRSP	196	V
Pyoderma	Dog	MRSP	339	NT
Pyoderma	Dog	MRSP	400	II-III
Pyoderma	Dog	MRSP	400	II-III
Surgical site infec.	Dog	MRSP	400	II-III
Pyoderma	Dog	MRSP	342	IV
Pyoderma	Dog	MRSP	342	IV

(c)



Cat	ST5	Staphylococcus epidermidis	IV
Cat	ST5	Staphylococcus epidermidis	NT
Cat	ST2	Staphylococcus epidermidis	NT
Dog	ST190	Staphylococcus epidermidis	NT
Dog		Staphylococcus epidermidis	II
Cat	ST20	Staphylococcus epidermidis	NT
Cat	ST2	Staphylococcus epidermidis	NT
Cat	ST35	Staphylococcus epidermidis	NT
Dog	ST57	Staphylococcus epidermidis	IV
Cat	ST23	Staphylococcus epidermidis	IV

(d)



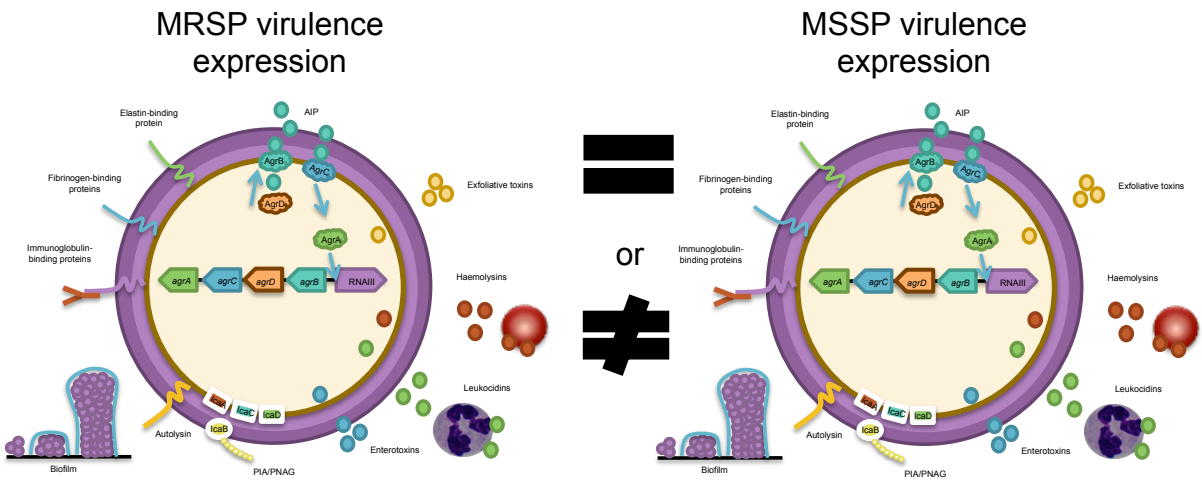
Dog	Staphylococcus haemolyticus	V
Dog	Staphylococcus haemolyticus	NT
Dog	Staphylococcus haemolyticus	V
Dog	Staphylococcus haemolyticus	NT
Dog	Staphylococcus haemolyticus	V
Dog	Staphylococcus haemolyticus	V
Cat	Staphylococcus haemolyticus	NT
Dog	Staphylococcus haemolyticus	V

3.3 Part 3 – The interaction between *Staphylococcus pseudintermedius* and dogs

3.3.1 Comparative analysis of the virulence characteristics of methicillin–resistant and –susceptible *Staphylococcus pseudintermedius* strains isolated from small animals: a RNA-Seq-based transcriptome analysis

Paper published in *Antimicrobial Agents and Chemotherapy*

Couto, N., Belas, A., Oliveira, M., Almeida, P., Clemente, C. & Pomba, C. (2015). Comparative RNA-seq-Based Transcriptome Analysis of the Virulence Characteristics of Methicillin-Resistant and -Susceptible *Staphylococcus pseudintermedius* Strains Isolated from Small Animals. *Antimicrobial Agents and Chemotherapy*, pii:AAC.01907-15.



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Comparative RNA-seq-Based Transcriptome Analysis of the Virulence Characteristics of Methicillin-Resistant and -Susceptible *Staphylococcus pseudintermedius* Strains Isolated from Small Animals

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Staphylococcus pseudintermedius **is often associated with pyoderma, which can turn into a life-threatening disease. The dissemination of highly resistant isolates has occurred in the last 10 years and has challenged antimicrobial treatment of these infections considerably. We have compared the carriage of virulence genes and biofilm formation between methicillin-resistant and methicillin-susceptible *S. pseudintermedius* (MRSP and MSSP, respectively) isolates and their *in vitro* gene expression profiles by transcriptome sequencing (RNA-seq). Isolates were relatively unevenly distributed among the four *agr* groups, and *agr* type III predominated in MRSP. Five virulence genes were detected in all isolates. Only the *spsO* gene was significantly associated with MSSP isolates ($P = 0.04$). All isolates produced biofilm in brain heart infusion broth (BHIB)–4% NaCl. MSSP isolates produced more biofilm on BHIB and BHIB–1% glucose media than MRSP isolates ($P = 0.03$ and $P = 0.02$, respectively). Virulence genes encoding surface proteins and toxins (*spsA*, *spsB*, *spsD*, *spsK*, *spsL*, *spsN*, *nucC*, *coa*, and *luk-I*) and also prophage genes (encoding phage capsid protein, phage infection protein, two phage portal proteins and a phage-like protein) were highly expressed in the MRSP isolate (compared with the MSSP isolate), suggesting they may play a role in the rapid and widespread dissemination of MRSP. This study indicates that MRSP may upregulate surface proteins, which may increase the adherence of MRSP isolates (especially sequence type 71 [ST71]) to corneocytes. MSSP isolates may have an increased ability to form biofilm under acidic circumstances, through upregulation of the entire *arc* operon. Complete understanding of *S. pseudintermedius* pathogenesis and host-pathogen signal interaction during infections is critical for the treatment and prevention of *S. pseudintermedius* infections.**

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) isolates have emerged as one of the leading causes of infectious diseases (including pyoderma, otitis and urinary tract infections) in companion animals, accounting for 20% to 47% of all clinical *S. pseudintermedius* isolates from dogs and cats (1). Moreover, some MRSP isolates are resistant to the antimicrobials regularly used for treatment (β-lactams, fluoroquinolones, tetracyclines, lincosamides, and potentiated sulfonamides) in small animal practice (1, 2). The *mecA* gene, encoding resistance to β-lactams, has been acquired by several *S. pseudintermedius* clonal lineages on independent occasions; however, two clones, MRSP ST68-SCC*mec* V and MRSP ST71-SCC*mec* II-III, are the dominant ones and have spread globally (1, 3, 4). This dissemination was rapid, but the reasons for the fast emergence and success of these lineages are not fully understood (2). Genomic and proteomic studies conducted in the last few years are giving the first clues on the pathways by which MRSP isolates have become successful. A recent genomic report suggested that multidrug resistance evolved rapidly in MRSP due to the acquisition of a very limited number of mobile genetic elements and mutations (1). Thus, the use of different antimicrobial classes coselected for the spread and emergence of the multidrug-resistant MRSP isolates (1). The frequent carriage of prophages in the MRSP sequence type 71 (ST71) and ST68 genomes suggested they have a role in the fitness of MRSP and that the predominant transfer of genetic material in these isolates is through bacteriophage transduction, rather than plasmid conjugation, as happens in methicillin-resistant *Staphylococcus aureus* (MRSA) (1). MRSP isolates are able to produce biofilm, and MRSP ST71 isolates, in particular, are better biofilm producers than other MRSP clones (5, 6). The *icaA* gene can be significantly upregulated in biofilm samples, suggesting a role in the biofilm production by *S. pseudintermedius* (7). The ability to form biofilm may play an important role in the pathophysiology of bacterial infections and can be related to survival and persistence of *S. pseudintermedius*, namely, MRSP, in the environment (5, 6). The MRSP ST71 isolates also show greater adherence to corneocytes than MRSP non-ST71 and methicillin-susceptible *S. pseudintermedius* (MSSP) isolates, and thus it has been suggested that the enhanced adherence of ST71 might be a factor contributing to the epidemiological success of this MRSP lineage (2). Furthermore, an MRSP ST71 isolate of human origin adhered evenly well to canine and human corneocytes, implying that MRSP ST71 may also be capable of adapting to human skin (2). Two proteins, SpsD and SpsO, can mediate adherence to canine corneocytes (8); however, the genetic factors responsible for the enhanced *in vitro* adherence of MRSP ST71 are not yet known (2).

In order to understand the epidemiological success of MRSP

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isolates, our goal was to understand if the phenotypes (biofilm) and genotypes (virulence genes) related to virulence factors were different between MRSP and methicillin-susceptible *S. pseudintermedius* (MSSP) isolates. Furthermore, we compared the *in vitro* transcriptional profiles by transcriptome sequencing (RNA-seq) of one MRSP isolate and one MSSP isolate to test the hypothesis that MRSP could have altered expression of virulence genes, by comparison with MSSP, which could have contributed to its rapid spread.

MATERIALS AND METHODS

Genotypic characterization of the MRSP and MSSP isolates. Twenty-one consecutive methicillin-resistant *S. pseudintermedius* (MRSP) isolates obtained over a 7-year period from 2007 to 2014 were included in the study. Twenty-one methicillin-susceptible *S. pseudintermedius* (MSSP) isolates matched in terms of isolation year, isolation site, and host were also included. These isolates were from 18 asymptomatic carriers (9 with MRSP and 9 with MSSP), 12 patients with pyoderma (6 with MRSP and 6 with MSSP), 6 patients with urinary tract infection (3 with MRSP and 3 with MSSP), 5 patients with otitis (2 with MRSP and 3 with MSSP), and 1 patient with a surgical site infection (MRSP). Five isolates were from cats, and 37 were from dogs. Isolates were characterized by multilocus sequence typing (MLST) (9). The eBURST algorithm identified groups of related sequence types (ST) (10).

Specific sequences for virulence genes involved in biofilm formation (*bap*, *icaA*, *icaB*, *icaC*, and *icaD*), enterotoxin production (*se-int*, sec_{canine}, and *seh*), host adherence (*ebpS*, *spsD*, *spsL*, and *spsO*), and toxin production (*lukS*, *lukF*, *siet*, *speta*, *expA*, and *expB*) were detected by PCR on a Mastercycle thermocycler (Eppendorf, New York) with the primers, product sizes, and annealing temperatures shown in Table S1 in the supplemental material (11–16). The primers designed in this study were generated using the Primer-BLAST tool from NCBI. All PCR products were analyzed by electrophoresis through 1.2% agarose gels (NZYTech, Lisbon, Portugal). The primers agrD-F (5′-GGG GTA TTA TTA CAA TCA TTC -3′) and agrD-R (5′-CTG ATG CGA AAA TAA AGG ATT G -3′) (STABvida, Monte da Caparica, Portugal) were used as previously described to amplify a 300-bp *agr* fragment encompassing the 3′ end of *agrB*, all of *agrD*, and the 5′ end of *agrC*. Amplification was carried out on a Mastercycler thermocycler (Eppendorf) under the following conditions: an initial 5-min denaturation step at 94°C, followed by 30 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 45°C, and 1 min of extension at 72°C, and a final extension step at 72°C for 10 min. The PCR products were purified by using NZYGelpure (NZYTech) and sequenced with the same primers used for the PCRs (STABvida). The 42 isolates were assigned to one of the four *agr* groups by comparing the predicted product of the *agrD* gene and the N-terminal half of the AgrC with those of four control isolates (GenBank accession no. EU157336, EU157366, EU157334, and EU157330).

Biofilm-producing ability on polystyrene. The capacity of the isolates to form biofilm was investigated by a method described by Stepanovic and colleagues (17) and Pettit and colleagues (18) with minor modifications, and was determined by the ability of *S. pseudintermedius* isolates to adhere to 96-well polystyrene microtiter plates (Greiner bio-one, Frickenhausen, Germany). In brief, the study was carried out using brain heart infusion broth (BHIB [Biokar]), BHIB with 4% NaCl, and BHIB with 1% glucose as the growth media. The plates were incubated at 37°C for 24 h. Following incubation, the alamarBlue solution was added to each well. After 30 min at room temperature, the optical densities at 570 nm (OD_{570}) were measured. *Staphylococcus epidermidis* strain RP62A (ATCC 35984) was used as a positive control. We defined the cutoff OD (OD_C) for the microtiter plate test as 3 standard deviations (SD) above the mean OD of the negative control as described previously (17). All isolates were classified into the following categories based upon the ODs of bacterial films (17): nonadherent, $OD \leq OD_C$; weakly adherent, $OD_C < OD \leq 2\times$ ODC; moderately adherent, $2\times OD_C < OD \leq 4\times OD_C$; or strongly adherent, $OD \geq 4\times OD_C$.

RNA isolation, sequencing and gene expression analyses. To test the hypothesis that MRSP and MSSP isolates differ in their expression of virulence genes, we compared the *in vitro* transcriptional profiles of a clinical MRSP isolate and a clinical MSSP isolate using RNA-seq. We attempted to choose 2 representative isolates from the *S. pseudintermedius* collection. The isolates were obtained from skin swabs of dogs with pyoderma (the most frequent clinical specimen from which *S. pseudintermedius* was isolated), they were obtained during the same period of time, were *agr* type III (the most frequent *agr* type found in this study), at least one was ST71 (the most frequent ST found in this study), and they had similar virulence profiles (considering virulence genes tested by PCR). Bacterial cells were grown until the mid-log-phase of growth (OD_{600} of 0.5), since it has been shown that the majority of surface proteins are produced during this phase (19). RNA was isolated using the RNeasy kit from Qiagen (Hilden, Germany). Briefly, 2×10^8 cells were removed from growing cultures, 2 volumes of RNAprotect bacterial reagent (Qiagen) was added, and the mixture was incubated for 5 min at room temperature. Cells were then centrifuged and incubated with TE buffer (30 mM Tris-Cl, 1 mM EDTA, pH 8.0 [Sigma]) containing 0.5-mg/ml lysostaphin (Sigma) and 15-mg/ml lysozyme (Sigma) for 20 min at 37°C. Proteinase K (20 mg/ml [Sigma]) was added, and the mixture was incubated for 10 min. After this, the procedure was carried out according to the manufacturer's specifications. The purified RNA was quantified using a NanoDrop spectrometer (ThermoScientific). RNA quality was assessed by visualization on an agarose gel. The rRNA was removed using the MICROBExpress kit (Ambion). RNA quality was then evaluated on a ByoAnalyzer (Agilent). Bacterial mRNA was fragmented (yield fragments were in the size range of 200 to 250 bp), and the double-stranded cDNA was generated using the Ion Total RNA-seq kit v2 (Life Technologies, Thermo Fisher Scientific) according to the manufacturer's instructions. The samples were sequenced using the Ion PGM (Life Technologies, Thermo Fisher Scientific) sequencer at STABvida.

Mapping to the reference genomes and normalization of gene expression were performed by CLC Genomics Workbench v8.0.1. RNA-seq reads were aligned with the three available *S. pseudintermedius* reference genomes ED99 (ST25, *agr* type III, lacks *spsF*, *spsO*, and *spsQ*), HKU10-03 (ST308, *agr* type III, lacks *nanB*), and E140 (ST71, *agr* type III, lacks *nanB*, *lukF*, and *lukS*) (RefSeq accession no. CP002478, CP002439 and ANOI01000001, respectively). Gene expression was normalized by calculating reads per kilobase per million mapped reads (RKPM), given by dividing the total number of reads by the number of mapped reads (in millions) × the length in kilobases (20).

Differentially expressed genes were identified using Baggerly's test (binomial test), which compares the proportions of counts in a group of samples against those of another group of samples (21) with a false discovery rate (FDR) correction applied (22). Genes with an adjusted *P* value of ≤0.05 were identified as being differentially expressed. This study focused particularly in the expression of virulence genes, but expression of other relevant genes (e.g., antimicrobial resistance genes) was also evaluated.

Statistical analysis. All data analysis was carried out using IBM SPSS Statistics version 20.0 (IBM, New York). Differences between the two groups MRSP and MSSP were calculated by Fisher's exact test for categorical comparisons and Student's *t* test for continuous outcome. A *P* value of ≤0.05 was considered statistically significant.

RESULTS

The results of MLST are shown in Table 1. The MSSP isolates were divided into 21 different STs, while 15 MRSP isolates were assigned to ST71, 3 to ST203, 1 to ST196, 1 to ST213, and 1 to ST195. Yet ST203 and ST195 belonged to clonal complex 71 (CC71), as detected by eBURST analysis. Equally, ST196 and ST213 differed by only one allele and belonged to CC196.

TABLE 1 Epidemiological characteristics of the MRSP and MSSP isolates used in this study

agr type	ST (no. of isolates)		
	MSSP (21)	MRSP (21)	*P* value[a]
All			0.025
I	ST207 (1), ST215 (1)	No STs	>0.05
II	ST201 (1), ST205 (1), ST206 (1), ST209 (1), ST217 (1)	ST196 (1), ST213 (1)	>0.05
III	ST17 (1), ST197 (1), ST199 (1), ST200 (1), ST202 (1), ST204 (1), ST210 (1), ST211 (1), ST212 (1), ST214 (1), ST379 (1)	ST71 (15), ST195 (1), ST203 (3)	0.014
IV	ST198 (1), ST208 (1), ST216 (1)	No STs	>0.05

[a] Shown are *P* values for differences between MRSP and MSSP.

TABLE 2 Virulence traits of the MRSP and MSSP isolates

Virulence gene[a]	No. of isolates with virulence gene shown			
	Total ($n = 42$)	MRSP ($n = 21$)	MSSP ($n = 21$)	*P* value
expA	0	0	0	>0.05
expB	3	0	3	>0.05
luk-I	40	19	21	>0.05
sec_{canine}	2	0	2	>0.05
seh	0	0	0	>0.05
spsD	4	1	3	>0.05
spsO	8	1	7	0.04

[a] The genes *ebpS*, *se-int*, *siet*, *speta*, and *spsL* and the *ica* operon were positive in all isolates and were not included in this table.

All isolates were classified as part of one of the four *agr* groups, and the distribution was highly uneven, with 2 isolates belonging to *agr* group I, 7 belonging to group II, 30 belonging to group III, and 3 belonging to group IV (Table 1). There was a significant difference in the *agr* groups' distribution between MRSP and MSSP ($P = 0.025$), with allele III being significantly more associated with MRSP than with MSSP ($P = 0.014$).

The virulence genes detected in the MRSP and MSSP isolates are detailed in Table 2. Genes *se-int*, *speta*, *siet*, *spsL*, and *ebpS* were present in all 42 isolates. The genes *lukF* and *lukS*, encoding leukocidin Luk-I, were found in all isolates except for two MRSP isolates (ST196 and ST213). Gene *expB* was detected in only 3 isolates. Only two MSSP isolates carried the enterotoxin gene sec_{canine}. No isolates harbored genes *seh* and *expA*. Eight isolates carried the *spsO* gene, and by statistical analysis, this gene was significantly more associated with MSSP than with MRSP ($P = 0.04$). No differences were found between clinical isolates and isolates from carriage.

Results related to the biofilm-forming ability on polystyrene are shown in Table 3. All isolates produced biofilm in the BHIB–4% NaCl medium. Two isolates did not produce biofilm on BHIB, and nine isolates did not produce biofilm on BHIB–1% glucose. Biofilm production in the BHIB and BHIB–1% glucose media was significantly higher in MSSP than in MRSP isolates ($P = 0.03$ and $P = 0.02$, respectively), but there were no differences between clinical isolates and isolates from carriage. The *ica* genes were detected in all 42 isolates.

The numbers of mapped reads assigned by using each reference genome (ED99, HKU10-30, and E140) are shown in Table S2 in the supplemental material. Of these mapped reads, the number of *S. pseudintermedius* genes with altered expression also varied when using the three different reference genomes, as shown in Fig. 1. The MSSP isolate had higher expression in transcription of regulatory genes *agrB* and *agrD*. On the other hand, the MRSP isolate had higher transcription of regulatory genes *sigB*, *srrA*, *sarA*, *rot*, and the *saeRS* system. The signal transduction protein TRAP gene (*traP*) was also highly expressed. Considering genes encoding surface proteins, only one, *spsC*, encoding an autolysin, was highly expressed in the MSSP isolate, while 6, *spsA*, *spsB*, *spsD*, *spsK*, *spsL*, and *spsN*, were highly expressed in the MRSP isolate. The gamma-hemolysin component B gene (*hlgB*), both subunits of the *luk-I* gene (*lukF-I* and *lukS-I*), and the coagulase and thermonuclease genes (*coa* and *nucC*, respectively) were upregulated in the MRSP isolate. The *arc* genes (*arcA*, *arcB*, *arcC*, and *arcD*) were upregulated in the MSSP isolate. Several genes associated with antimicrobial resistance were highly expressed in the MRSP isolate: the *norA*, *gyrA* and *gyrB* genes associated with quinolone resistance, the *aadE* and the bifunctional *aacA-aphD* genes associated with aminoglycoside resistance, the *mecA*, *mecR1*, and *blaI* genes associated with β-lactam resistance, and the *tet*(M) gene associated with tetracycline resistance. The MRSP isolate upregulated several phage-associated genes (encoding phage capsid protein, phage infection protein, two phage portal proteins, and a phage-like protein), and an integrase gene located in the superantigen-encoding pathogenicity island SaPI (SPSINT_0063).

DISCUSSION

In the last 10 years, MRSP isolates have become highly frequent in clinical samples from infected animals and as colonizers of healthy ones (1). However, it is still not clear why MRSP isolates, especially certain lineages like ST71, have spread so quickly. To understand the rapid evolution that led to the dissemination of MRSP isolates, we assessed the virulence determinants present in a collection of MSSP and MRSP isolates and compared their abilities to form biofilm in 3 different media. Finally, we performed *in vitro* gene expression analysis and compared the levels of expression of one MSSP isolate and one MRSP isolate.

Analysis of the virulence genotype of the MRSP and MSSP isolates revealed a strong conservation of genes: five genes (*ebpS*, *se-int*, *siet*, *speta*, and *spsL*) were carried by all *S. pseudintermedius* isolates, and five genes (*expB*, *luk-I*, sec_{canine}, *spsD*, and *spsO*) were only present in some isolates. Two studies have reported the existence of some specific toxin genes (e.g., *coa*, *lip*, *geh*, *htrA*, *nuc*, *clpX*, *hlb*, *se-int*, *speta*, *spsA*, *spsB*, and *spsC*) present in several *S. pseudintermedius* isolates that might be important for the canine host tropism, in particular the skin (1, 23). However, variation was found in others (e.g., *spsF*, *spsO*, *spsP*, *spsQ*, *luk-I*, and *nanB*), suggesting that a difference in virulence factors in the core genome was probably lineage associated (1). For example, in one of these studies, the five ST71 isolates lacked the *nanB* and *lukF* and *-S* genes (1). Still, in our study and in a previous study conducted in Spain (24), all of the ST71 isolates carried the *lukF* and *-S* genes, suggesting that variation may also be related to the region of isolation. It would be interesting to collect a large collection of ST71 isolates from different countries to study these variations. In other lineages, however, this will be difficult to ascertain, since only a few isolates in each lineage have been reported so far.

The capacity of bacteria to form biofilms is an important viru-

TABLE 3 Overall results of the microtiter plate test according to the pattern of methicillin resistance in *S. pseudintermedius*

Medium	Mean OD_{570} ± SD	No. of isolates showing:			
		No adherence	Weak adherence	Moderate adherence	Strong adherence
MRSP					
BHIB	0.50 ± 0.052	1	5	15	0
BHIB + 1% glucose	0.28 ± 0.038	3	18	0	0
BHIB + 4% NaCl	0.44 ± 0.055	0	18	3	0
MSSP					
BHIB	0.57 ± 0.082	1	6	14	0
BHIB + 1% glucose	0.35 ± 0.081	6	15	0	0
BHIB + 4% NaCl	0.45 ± 0.098	0	13	8	0

lence factor not only in the development of device-related infections but also in a range of chronic infections (23). This capacity might further complicate the treatment of already challenging infections due to the decrease in effectiveness of antimicrobials on biofilms (5). In one study, all *S. pseudintermedius* isolates produced biofilms, suggesting that biofilm production might be essential for the pathogenicity of *S. pseudintermedius* (6). Yet, the study failed to find differences in the biofilm formation between MRSP and MSSP isolates. The number of MSSP isolates that was studied was low, and the authors suggested that further experiments with a larger number of isolates were warranted (6). By using a larger set of isolates, we observed that biofilm production in the BHIB and BHIB–1% glucose media was significantly higher in MSSP than in MRSP isolates. This is a phenomenon that has been observed in *S. aureus*, when comparing methicillin-resistant and methicillin-susceptible isolates, and is due to different triggering mechanisms leading to biofilm formation, including *ica*-dependent and -independent mechanisms (25). In our study, all isolates produced biofilm, and all were positive for the *ica* genes, suggesting this operon has a crucial role in biofilm formation. However, the mechanisms triggering the higher biofilm production in the BHIB and BHIB–1% glucose media by MSSP strains remain unknown. One clue to this occurrence may be related to the upregulation of the entire *arc* operon in the MSSP isolate studied here. A similar operon has been found in other staphylococcal species, and in *S. aureus*, *arcA* (which belongs to the *arc* operon) encodes an arginine deaminase, which allows for enhanced survival in acidic environments (26). The upregulation of this operon may improve survival and promote biofilm formation of MSSP in acidic circumstances, such as in BHIB medium with glucose (which has a more acidic pH than BHIB medium alone or with NaCl).

During the early emergence of community-acquired MRSA, the USA300 (ST8) lineage disseminated rapidly and was considered hypervirulent, compared with lineages like MRSA USA400 (ST1) (26). However, USA300 does not contain many more virulence genes than USA400, but it does have an alteration in the expression of regulatory genes and increased expression of certain virulence genes (26). By microarray analysis, USA300 displayed an increased expression of genes encoding cell envelope proteins (including lipoproteins and superantigen-like proteins), genes residing in the prophage ϕSa3usa, several genes contained in pathogenicity islands vSAα and vSAß, genes encoding proteases, and the gene encoding the IgG binding protein Sbi (26). Interestingly our MRSP isolate also had increased expression of several genes, including *spsK*, which encodes the IgG binding protein Sbi, the toxin genes *nucC* and *coa*, prophage genes, and several virulence regulatory genes, including *saeRS*. The higher expression of the prophage genes might be one of the factors contributing to the rapid dissemination of MRSP, particularly ST71 isolates. The higher

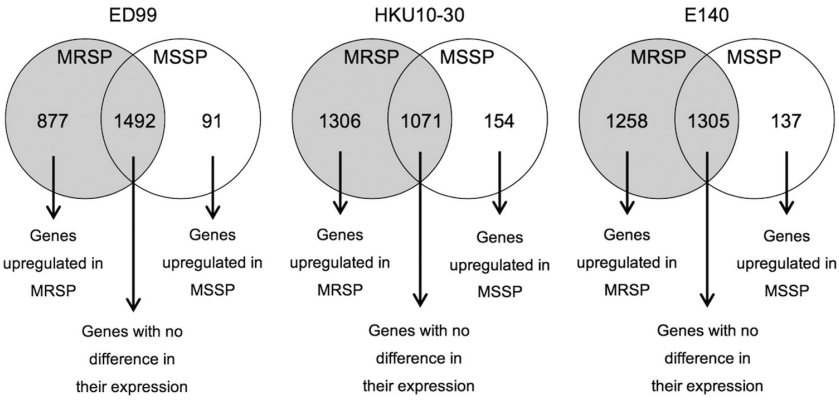

FIG 1 Number of *S. pseudintermedius* genes with altered expression identified by Baggerly's test with the FDR correction applied, using the three different reference genomes available ED99 (ST25, *agr* III), HKU10-30 (ST308, *agr* III), and E140 (ST71, *agr* III).

expression of the genes *spsD* and *spsL* (encoding fibronectin-binding proteins able to adhere to the extracellular matrix) found in this study may explain the higher adherence of MRSP ST71 isolates to corneocytes previously detected (2). We observed a very different expression of virulence regulatory genes between the two isolates, with *agr* highly expressed in MSSP and *saeRS* highly expressed in MRSP. This may explain the differences observed in the expression of the genes encoding surface proteins and toxins.

Among the most important bacterial defenses against uptake of foreign DNA are the restriction-modification (R-M) systems (27). These systems, comprising restriction endonucleases and methyltransferases, recognize and modify specific DNA sequences, protecting "own" DNA from restriction while eliminating potentially harmful foreign DNA (27). In *S. pseudintermedius*, type I R-M systems have been recognized, including one that was carried on all SCC*mec* II and III elements of MRSP ST71 (1). One study suggested that MRSP isolates were not more efficient or inefficient than MSSP isolates in acquiring mobile genetic elements due to the wide distribution of the type I and type II R-M systems in *S. pseudintermedius* isolates (1). In our study, however, we found that the type I restriction-modification system restriction subunit R (*hsdR*) was highly expressed in the MSSP isolate, suggesting it blocks DNA horizontal gene transfer into methicillin-susceptible isolates. Lower expression of subunit R in the MRSP isolate could also suggest a more efficient way of acquiring mobile genetic elements. In fact, it has been shown that MRSP genomes carry more prophages than MSSP isolates. Our results showed that the MRSP isolate also upregulates several phage-associated genes, which could be linked to the upregulation of the integrase located in the superantigen-encoding pathogenicity island, SaPI. The upregulation of prophage particles is also concordant with the suggestion that transfer in MRSP is predominantly made by transduction (1).

In summary, this is the first study to document the global transcription differences between the MSSP and MRSP isolates during *in vitro* growth. This study indicates that MRSP may upregulate surface proteins, which may increase the adherence of MRSP isolates (especially ST71) to corneocytes. Although MRSP and MSSP have the capacity to form biofilm, MSSP may have an increased ability to form biofilm under acidic circumstances, through upregulation of the entire *arc* operon. Complete understanding of *S. pseudintermedius* pathogenesis and host-pathogen signal interaction during infections is critical for the treatment and prevention of *S. pseudintermedius* infections.

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3.3.2 Identification of vaccine candidate antigens of *Staphylococcus pseudintermedius* by whole proteome characterization and serological proteome analysis

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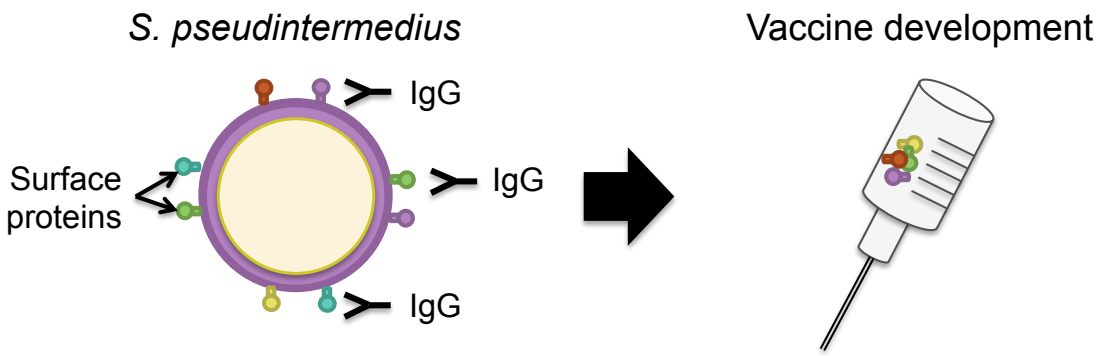

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Identification of vaccine candidate antigens of *Staphylococcus pseudintermedius* by whole proteome characterization and serological proteomic analyses

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ABSTRACT

The recent emergence of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has complicated considerably the treatment of infections caused by these bacteria. Therefore new treatment strategies are urgently needed, namely through the development of vaccines towards the control of bacterial infections. Our study describes an extensive characterization of the proteome of *S. pseudintermedius* through a 2-DE MALDI-TOF/TOF approach, followed by SERological Proteome Analysis (SERPA) to identify potential vaccine candidate antigens. We were able to identify 361 unique proteins, of which 39 are surface proteins. In order to assess the immunogenic potential of *S. pseudintermedius* proteins, a Western blot analysis of two-dimensional gels was carried out with serum from healthy dogs, dogs with atopic dermatitis infected and not infected with *S. pseudintermedius*. Only immunogenic areas detected by ≥50% of the dogs with atopic dermatitis infected with *S. pseudintermedius* sera and by <50% of the healthy dogs sera were excised and identified from Coomassie-colloidal stained gels. The areas identified by IgE were not considered as vaccine targets, because those proteins could induce hypersensitivity. We were able to identify 13 unique proteins after *in-gel* digestion of selected protein gel spots, with 4 antigenic proteins showing promising features for vaccine development. No specific antibodies were identified in the dogs with atopic dermatitis not infected with *S. pseudintermedius* sera that could contribute to prevention of infection. The SERPA approach employed in this study revealed novel candidate therapeutic targets for the control of *S. pseudintermedius* infections.

1. Introduction

Staphylococcus pseudintermedius is a Gram-positive coccus, which belongs to the *Staphylococcus intermedius* group (SIG) [1]. Like *Staphylococcus aureus*, *S. pseudintermedius* are normally harmless and colonize the skin and mucous membranes of animals [1]. However, they can also be pathogenic by overcoming the hosts' immune system and of clinical interest because they cause a variety of infections among small animals, especially dogs, causing high morbidity [1]. One of the most common diseases caused by *S. pseudintermedius* is pyoderma in dogs. Pyoderma is usually secondary to atopic dermatitis, a pruritic allergic skin disease that results in disrupted skin barrier and predisposition for secondary *S. pseudintermedius* infections [2]. *S. pseudintermedius* produces a wide range of virulence factors, especially proteins. However, not much is known about the role of these proteins during infection [3]. In *S. aureus*, homologue proteins are produced in a cell density-dependent manner, through the *agr* system [4]. To initiate infection, *S. aureus* synthesizes surface proteins, like adhesins, that bind extracellular-matrix molecules and promote colonization [4]. Once colonization has been established the bacteria multiply, activating a density-sensing mechanism that stimulates the production of exoproteins, like exfoliative toxins and leukocidins, therefore promoting the spread of bacteria to other sites [4]. It has been hypothesized that the *agr* system of *S. pseudintermedius* may play a comparable role in the pathogenesis of these bacteria in canine pyoderma [3].

Pyoderma is usually treated with antimicrobials [1]. The selective pressure imposed by the long-term administration of antimicrobials can lead to the development of bacterial resistance that could potentially be transmitted to human pathogens, like *S. aureus*. The recent emergence of methicillin-resistant *S. pseudintermedius* (MRSP) has complicated considerably the treatment of infections caused by these bacteria. MRSP have become virtually resistant to all the antimicrobials approved for administration in companion animals, which has led to ethical concerns about the use of antimicrobials classified by the World Health Organization as "critically important" for human medicine [1]. In this way new treatment strategies are urgently needed and one way of controlling bacterial infections is through the development of vaccines.

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The "omics" era (genomics, transcriptomics and proteomics) has brought new tools that provide information about cell's genetic background, their potential regulatory mechanisms and biological activity of proteins [5]. Proteomics has the advantage of dealing directly with proteins, which are the main players of life processes [6]. Proteome characterization of bacterial species, especially of their surface-exposed protein fractions, can lead to the identification of vaccine candidates, which can then be tested for their ability to induce protective immunity or as passive immunization targets [5,7,8]. This can be accomplished in silico, using bioinformatics tools, and/or by combining proteomics with serological analysis (SERPA — SERological Proteome Analysis) [7–14]. This approach has enabled the identification of vaccine candidates against *S. aureus* [12–14].

The aim of this study was to characterize the proteome of *S. pseudintermedius* through the 2-DE MALDI-TOF/TOF approach and subsequently use a SERPA approach, using immunoblots followed by MS protein identification, to depict potential vaccine candidate antigens. To design potent and generally applicable subunit vaccines, it is necessary to identify those antigens that are recognizable on a wide patient population during infection [9]. For this reason we used serum from dogs with atopic dermatitis and recurrently infected with *S. pseudintermedius* (ADI) and discarded those proteins identified by healthy dogs (H), to detect proteins only expressed during infection. We used a third group, dogs with atopic dermatitis and not recurrently infected with *S. pseudintermedius* (ADH), in an attempt to identify antigenic proteins for which specific antibodies were missing or underrepresented in infected patients that could contribute to prevention of infection.

2. Material and methods

2.1. Bacterial strain and culture conditions

S. pseudintermedius strain FMV5819/10 was isolated from a dog with deep pyoderma presented to the Dermatology Service of the Teaching Hospital FMV-UL. This strain belongs to sequence type 71 and has an SCC*mec* II–III, representing the European MRSP clone ST71-II–III [16]. An overnight culture was inoculated into brain–heart infusion broth (BHIB, Biokar® Solabia Group, Pantin, France) until the mid-exponential phase was reached, corresponding to an OD_{600} of 0.4–0.5.

2.2. Protein fractionation

The fractionation protocol was optimized based on previously published protocols [12,17] and is summarized in Fig. 1. Briefly, cells were harvested by centrifugation at 8000 ×*g* for 5 min at 4 °C. Cells were washed twice with isotonic buffer (10 mM Tris–HCl pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTE, 1.1 M saccharose and protease inhibitors; Sigma, St. Louis, United States of America), followed by resuspension in isotonic buffer with 100 μg/ml of lysostaphin (Sigma). The suspension was incubated at 37 °C for 35 min. Then protoplasts were sedimented by centrifugation at 8000 ×*g* for 30 min at 4 °C. The supernatant containing cell wall proteins (S1) was precipitated with trichloroacetic acid 20% (v/v) with 2-mercaptoethanol 0.14% (v/v) (Sigma) for 1 h at 4 °C and harvested at 13,000 ×*g* for 30 min at 4 °C. The precipitate was rinsed two times with acetone and centrifuged at 13,000 ×*g* for 9 min at 4 °C.

Unbroken protoplasts were re-suspended in DNase I (20 μg/ml) and RNase A (20 μg/ml) (Sigma) for 10 min at room temperature and then centrifuged at 3500 ×*g* for 30 min at 4 °C. The pellet containing cellular debris was discarded and the supernatant was centrifuged at 100,000 ×*g* for 9 min at 4 °C. From this centrifugation, we recovered proteins in the pellet (P) and in the supernatant (S2), which were precipitated in 50 mM ammonium bicarbonate (Sigma) and 2,2,2-trifluoroethanol/chloroform (TFE/$CHCl_3$) 1:1 (v/v) mixture (Sigma) and maintained at 0 °C for 1 h with periodic vortexing. A final centrifugation at 10,000 ×*g* for 4 min at 4 °C allowed the separation of both mixtures (P and S2) into three phases: aqueous, insoluble and chloroformed. For both mixtures, aqueous (PFA and S2FA) and insoluble (PFI and S2FI) phases were recovered for further analysis. Chloroform phases were discarded due to the low amount of protein present. All protein fractions (S1, PFI, PFA, S2F1 and S2FA) were dried using a speedvac (ThermoFisher) and stored at −20 °C until use. The obtained pellets were resuspended in a buffer containing 7 M urea (Sigma), 2 M thiourea (Sigma), 30 mM Tris (Sigma) and 4% CHAPS (Sigma).

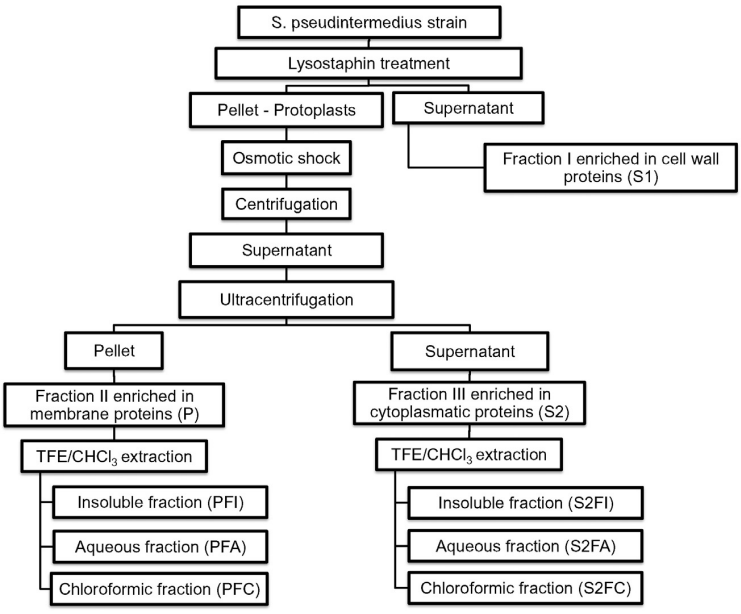

Fig. 1. Protein fractionation procedure applied to *S. pseudintermedius* FMV5819/10.

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2.3. 2-DE and image analysis

The *S. pseudintermedius* proteins were separated in five different cellular fractions. Fraction I was enriched with cell wall proteins (S1), insoluble Fraction IIa enriched with membrane insoluble proteins (PFI), aqueous Fraction IIb enriched with membrane water-soluble proteins (PFA), insoluble Fraction IIIa enriched with cytoplasmic insoluble proteins (S2FI), and finally, aqueous Fraction IIIb enriched with cytoplasmatic water-soluble proteins (S2FA). The total protein of three biological replicates of all the fractions was quantified with 2D-Quant kit (GE healthcare Europe GmbH, Freiburg, Germany) and separated by two-dimensional electrophoresis.

For fractions I, IIa and IIIa, the first-dimension was performed using 24 cm immobilized 3–10 NL pH gradient (GE healthcare). The IPG strips were loaded with 500 μg of protein and submitted to active rehydration, in a rehydration buffer containing 0.5% (w/v) ampholytes (Amersham) and 50 mM DTE (Sigma), for 20 h at low voltage (30 V). Isoelectric focusing (IEF) was carried out with IPGphor system (GE healthcare) using the following program: 2 h 100 V, 1 h 300 V, 2 h 500 V, 2 h 1000 V, gradient from 1000 V to 3000 V, 3 h 3000 V, 3.5 h gradient from 3000 V to 8000 V and 8 h 8000 V, completing the run to a total voltage of 98.4 kVh, 101.1 kVh and 98.5 kVh for fractions I, IIa and IIIa, respectively. The first dimensional run was performed at 20 °C with a maximum current of 50 μA/strip. Immediately after the first-dimension run, the samples were reduced at room temperature by gentle agitation for 9 min in equilibration buffer (6 M Urea, 2% (w/v) SDS, 50 mM Tris pH 8.8, 0.02% (w/v) bromophenol blue, 30% (v/v) glycerol; Sigma) supplemented with 2% (w/v) DTE (Sigma), followed by alkylation for 9 min in equilibration buffer supplemented with 3% (w/v) iodoacetamide. SDS-PAGE was done using 12.5% polyacrylamide gels and the separation was run following program: 1 h at 100 V, 10 mA/gel and 1 W/gel, and 4 h at 600 V, 38 mA/gel and 17 W/gel. For less complex protein samples, namely the water-soluble proteins' fractions (IIb and IIIb), the isoelectric focusing was performed using 7 cm immobilized 3–10 NL pH gradient (GE healthcare). The IPG strips were submitted to active rehydration for 12 h at low voltage (30 V) and then loaded with 50 μg of protein. The first dimensional running followed program: 2 h 100 V, 1 h 300 V, 2 h 500 V, 2 h 1000 V, gradient from 1000 V to 3000 V, 3 h 3000 V, 3.5 h gradient from 3000 V to 8000 V and 8 h 8000 V, completing the run at a total voltage of 22.0 kVh.

The gels were stained with Coomassie-colloidal (Sigma). The images were acquired in the Laser-based scanner FLA-5100 (FujiFilm, Valhalla, United States of America) using 532 nm-excitation laser, and were analyzed using Progenesis SameSpots v4.5 (NonLinear Dynamics, Newcastle, United Kingdom). The three images, each for one biological triplicate, were aligned automatically and a spot list was generated. The operator made the spot detection and filtering, and only spots found at least in two out of the three gels were considered. No statistical restriction analysis was done. Selected spots were excised from these gels, washed with water and 50% acetonitrile, dehydrated with 100% acetonitrile and vacuum-dried. Proteins were *in-gel* digested with 6.7 ng/μL of trypsin (Promega, Madison, United States of America). The tryptic peptides were acidified with 5% (v/v) formic acid, concentrated with POROS R2 microcolumns (GELoader tip, Eppendorf, Hamburg, Germany) and co-crystallized onto MALDI-TOF/TOF sample plates using the α-cyano-4-hydroxycinnamic acid (Sigma) matrix.

2.4. Protein identification by MALDI-TOF/TOF MS

Peptide mass spectra were acquired using a MALDI-TOF/TOF 4800 plus MS/MS (Applied Biosystems® Life Technologies, Carlsbad, United States of America). Data were acquired in positive MS reflector using a *PepMix1* (LaserBio Labs, Sophia-Antipolis, France) to calibrate the instrument. Each reflector MS spectrum was collected in a result-independent acquisition mode; using 750 shots per spectra in 800–4000 *m/z* range and fixed laser intensity to 3100 V. Fifteen of the strongest precursors were selected for MS/MS. MS/MS analyses were performed using CID (Collision Induced Dissociation) assisted with a collision energy of 1 kV and a gas pressure of 1×10^{-6} Torr. For each MS/MS spectrum, 1400 laser shots were collected, using fixed laser intensity of 4400 V. Processing and interpretation of MS and MS/MS spectra were performed with the 4000 Series Explored™ Software (Applied Biosystems® Life Technologies, Carlsbad, United States of America).

Protein identification was performed using tandem mass spectral data and MASCOT search engine using the MOWSE algorithm (Matrix Science, version 2.2.07, Boston, United States of America) on *S. pseudintermedius* database (5029 sequences; 1,481,467 residues) retrieved from NCBI (downloaded in August 2013). Searches included trypsin as digesting enzyme; peptide mass tolerance of 50 ppm; fragment mass tolerance of 0.5 Da and possible oxidation, carbomidomethylation or deaminidation as variable amino acid modifications with one missed cleavage. Peptides were only considered if the ion score indicated extensive homology ($p < 0.05$). Proteins were considered if the protein score indicated significant statistical confidence ($p < 0.05$). Protein identifications with only one matched peptide were considered if they were identified with >95% confidence.

2.5. Immunoblotting

The surface cellular proteins (cell wall and membrane protein fractions) were separated by 2-DE. The first dimension was performed in 7 cm immobilized 4–7 pH gradient loaded with 20 μg of protein, until a total voltage between 18 and 22 kVh was reached. All samples were then reduced in 2% (w/v) DTE, followed by alkylation with 3% (w/v) iodoacetamide. SDS-PAGE was done using 12% polyacrylamide gels (NuPAGE Bis–Tris Precast gels, Life Technologies® Thermo Fisher Scientific, Waltham, United States of America). Proteins were transferred onto PVDF membranes in a Xcell II TM Blot Module (Invitrogen® Thermo Fisher Scientific) at 30 V for 65 min. Membranes were washed and blocked for 1 h, with 10% for IgG and for IgE (w/v) Dry Non-Fat Milk (BioRad)/0.5% (v/v) Tween 20, and incubated with dogs' sera (1:10 000) overnight at 4 °C. Dog's serum from which *S. pseudintermedius* strain FMV5819/10 was isolated was used as a reference. Other sera were collected from healthy donors — H ($n = 13$) and atopic donors both with *S. pseudintermedius* recurrent pyoderma — ADI ($n = 16$) and without — ADH ($n = 8$). Each serum was incubated individually. The attending veterinarian diagnosed dogs with atopic dermatitis according to accepted criteria and after ruling out other causes of pruritus: a compatible history; clinical criteria strongly associated with the disease; exclusion of other pruritic skin diseases, with no response to an 8 week minimum trial consisting of either home-cooked single protein or commercial hydrolyzed protein diets and water, an 8 week veterinary-approved flea control regimen, and exclusion of sarcoptic mange by trial therapy and/or negative serology; and at least one positive reaction to a perennial allergen on intradermal or serological testing. Pyoderma was diagnosed by compatible cytology (neutrophils, cocci and neutrophils with phagocyte cocci) and isolation of *S. pseudintermedius*. Pyoderma was considered recurrent if the animal presented at least with two episodes.

After washing, specific binding of sera IgG and IgE was visualized by incubation with an anti-dog-IgG peroxidase conjugate (1:20 000) (Santa Cruz Biotechnology, Texas, United States of America) and anti-dog-IgE peroxidase conjugate (1:1000) (AbD Serotec® BioRad, Oxford, United Kingdom), respectively, and revealed in a ChemiDoc XRS + system (BioRad). Membranes were also stained with 0.1% (v/v) Ponceau S (Sigma). Highly immunogenic areas were compared between groups. Only immunogenic areas identified by ≥50% of the ADI sera and identified by <50% of the H sera were excised and identified from Coomassie-colloidal stained gels. The ADH sera were used in an attempt to identify antigenic proteins for which specific antibodies were missing or under-represented in infected patients that could contribute to prevention of infection. Matching signals from immunoblots and protein spots were

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done by comparing films with the image of the Ponceau S stained membrane and the Coomassie-colloidal stained preparative gel. Proteins were digested and identified as stated above.

2.6. Bioinformatic analysis

Bioinformatic analyses were performed using web-based servers. The functional role categories assigned were the ones used in the *S. aureus* NCTC 8325 annotation proposed by The Institute for Genomic Research [18]. Blast2go was used to find homologous sequences, when proteins were characterized as unknown. PSORTb v3.0 [19] was used to predict cellular localization of a protein. The program TMPred in ExPASy [20] identified the predicted transmembrane regions for proteins. Cleavable signal peptide predictions were performed from SignalP v4.1 trained on Gram-positive bacteria [21], PrediSi trained on Gram-positive [22] and TargetP v1.1 [23,24]. Prediction of non-classical secreted proteins, lacking a signal peptide, was performed using SecretomeP v2.0 [25].

3. Results and discussion

3.1. Fractionation of S. pseudintermedius FMV5819/10 proteins

To better characterize the proteins from *S. pseudintermedius* strain FMV5819/10, we performed a fractionation procedure based on previous protocols used in other *Staphylococcus* species. Fig. 2 and Supplementary Fig. 1 (with the Spots ID) show the 2-DE patterns of *S. pseudintermedius* FMV5819/10 protein fractions: cell wall associated, membrane and cytoplasmatic. The first fraction was obtained after lysostaphin treatment. This enzyme cleaves specifically the cross bridges of the staphylococcal peptidoglycan, maintaining the cellular membrane intact [26]. In this way, conceptually, only cell wall components are extracted, turning fraction I enriched in these proteins. However, it is unavoidable to have some degree of contamination with proteins from other cell compartments. In our study, most of the proteins, determined as cell wall associated or secreted, were found in Fraction I (Table 1), but several membrane and cytoplasmatic proteins were also detected. This could be due to lysostaphin concentration or incubation time used. We selected a concentration of 5 U and an incubation time of 30 min based on the findings of Nandakumar and colleagues for *S. aureus* [26]. However, different *Staphylococcus* species have different susceptibilities to lysostaphin, and so, lysostaphin may need less concentration and/or incubation time to lyse the peptidoglycan of *S. pseudintermedius*. Furthermore, the identification of intracellular proteins on the cell surface has been reported in *S. aureus*, *S. pseudintermedius* and other coagulase-negative staphylococci, and is due to alternative functions associated with the cell surface described for some cytoplasmatic proteins [27].

Fractions II and III were separately enriched in membrane and cytoplasmatic proteins, respectively, after separation of the two fractions by ultracentrifugation. From fraction II we recovered membrane proteins, lipoproteins, proteins interacting with membrane components and subunits of membrane-associated complexes (Table 1). Most proteins identified in fraction III were cytoplasmatic, however we found at least 5 proteins that were associated with the surface proteome (2 cell wall associated, 2 extracellular and 1 membrane protein), which could indicate that they have multiple subcellular locations or that they can transit between the cytosol and the surface compartments, depending on the physiological and/or environmental conditions.

Although a complete fractionation of the proteins could not be achieved, their identifications revealed that enrichment on the proteins of each cellular fraction was succeeded, which indicates that the established fractionation protocol was efficient and adequate for the characterization of *S. pseudintermedius* proteome.

3.2. Protein identification and in silico analysis of the cell wall associated, extracellular and membrane proteins encoded by S. pseudintermedius FMV5819/10

From a total 1317 gel spots excised we identified 361 unique proteins. The parameter values for the identification and subcellular localization predicted by PSORTb, TMPred, PrediSi, SignalP, SecretomeP and/or TargetP of all proteins are present in Supplementary Tables 1 and 2. As expected, most proteins were cytoplasmatic (72.2%), followed by proteins with unknown location (9.5%), ribosomal proteins (7.6%), membrane proteins (6.5%), extracellular proteins (2.5%) and finally proteins from the cell wall (1.6%). Yet 6 of these last proteins (17.1%) had detectable peptide signal cleavage sites identified by SignalP and SecretomeP and/or TargetP (Table 1). Ten proteins with unknown location were identified as secreted via unknown secretion system by SecretomeP, while one protein was also detected as secreted by SecretomeP and TargetP and had a predicted cleavage site at position 25 identified by PrediSi, but not by SignalP. All proteins identified as extracellular by PSORTb had predicted cleavage sites identified by all algorithms, except for one protein, which was only identified as secreted via unknown secretion system by SecretomeP.

Three proteins had sequenced motifs relevant for vaccine development. Three cell wall associated proteins (Fibronectin binding protein FnbB [SpsD], Uncharacterized protein (LPXTG-motif cell wall anchor domain protein) [SpsN], Immunoglobulin G binding protein A spa1 [SpsQ]) had a C-terminal LPXTG motif (Pfam: PF00746) and a concurrent YSIRK motif (Pfam: PF04650). The LPXTG motif is a hydrophobic domain, which is recognized and cleaved by an enzyme present in the staphylococcal membrane, sortase A (SrtA), and then attached to the peptidoglycan [28]. Since numerous microbial surface components recognizing adhesive matrix molecules (MSCRAMMS), which are usually proteins involved in tissue adhesion and immune evasion, have the LPXTG motif, SrtA has a fundamental role in the staphylococcal pathogenesis. The LPXTG motif or the SrtA could be potential targets to minimize strain's expression of adhesins and so inhibit the establishment of infection. The YSIRK motif is usually positioned within signal peptides and is required for the secretion of cell wall anchored surface proteins [29]. Although also regarded as a motif required for cell wall anchoring of surface proteins, it has been shown that this motif plays a much more efficient role in the secretion of these proteins, like protein A, then for the anchoring itself [29]. However, blocking this motif could inhibit the secretion of important immune evasion proteins, like the already mentioned protein A, which could in turn lead to an enhanced response by the cells of the immune system. As one would expect, the proteins with the LPXTG and YSIRK motifs also exhibited a Sec-dependent signal peptide. One protein classified as “with unknown subcellular location”, SpsK (predicted IgG-binding protein Sbi), has been previously described as cell wall associated in *S. pseudintermedius* [27], however PSORTb and InterProScan failed to identify any cell wall associated domains or motifs. Sbi in *S. aureus* also lacks the typical Gram-positive cell wall anchoring sequence LPXTG [30]. In fact this protein is mainly described as secreted in *S. aureus* [31], although it has been suggested that Sbi is associated with the bacterial surface through electrostatic interactions, or because it does have a predicted proline-rich cell wall-spanning segment normally found within cell-wall-spanning domains [30]. The Immunoglobulin G binding protein A spa1 (SpsQ) also had a Lysin motif (LysM) (Pfam: PF01476), a binding domain that allows non-covalent binding to the cell wall [17]. The LysM sequence is present singly or repeatedly in a large number of proteins of prokaryotes and eukaryotes [32]. This motif has been extensively studied due to the possibilities of employing the LysM domains for cell immobilization, for the display of peptides, proteins, or enzymes on (bacterial) surfaces as well as their utility in the development of novel vaccines [32]. The LysM motif has been used to produce antigen-to-LysM fusions, including bacterial, viral and parasitic antigens, which have been successfully attached to Gram-positive bacteria *Lactococcus lactis* [32]. This is how

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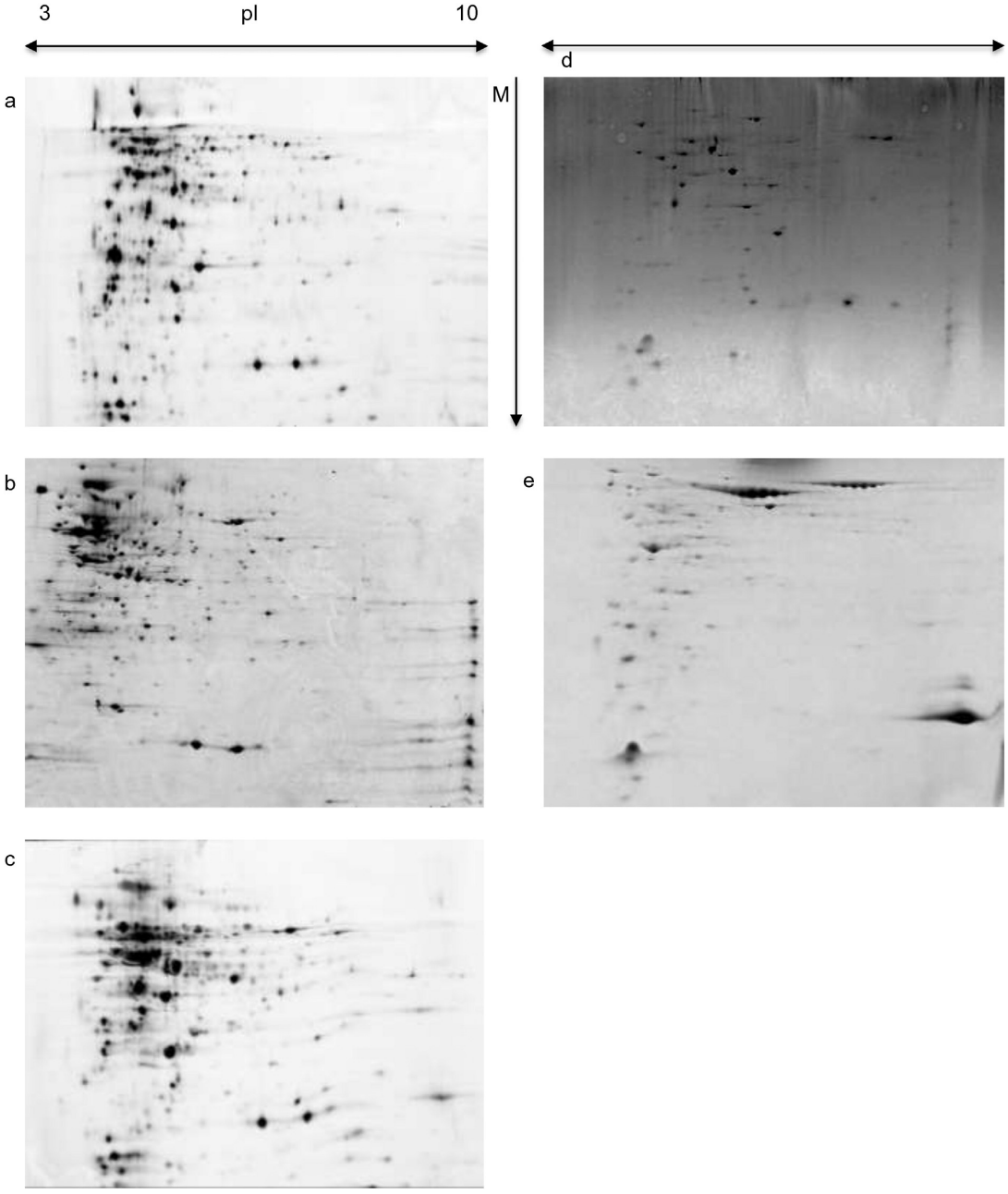

Fig. 2. 2-DE patterns of *S. pseudintermedius* FMV5819/10 protein fractions: a) cell wall associated; b) membrane (insoluble subfraction); c) cytoplasmatic (insoluble subfraction); d) membrane (soluble subfraction) and e) cytoplasmatic (soluble subfraction).

the novel adjuvant Gram-positive enhancer matrix (GEM) particles are produced [32]. The GEM particles produced by heating the *L. lactis* in acid are non-living, deprived of intact surface proteins and intracellular content, however the thick peptidoglycan cell wall remains intact and provides the structural rigidity to constitute the bacterial-shaped peptidoglycan spheres, referred to as GEM particles. Studies have demonstrated that antigens displayed on GEM particles induce higher immune response than antigen alone [32]. This is important when considering the development of a *S. pseudintermedius* vaccine.

The biological functions of unique identified proteins are described in Fig. 3. Proteins were mostly related with the energy metabolism (19.0%), with purines, pyrimidines, nucleosides and nucleotides (10.0%) and protein synthesis (10.0%). These biological functions are all related to the cell's basic metabolism. However, we also identified 2.0% of proteins associated with pathogenesis. Three main areas of *S. aureus* pathogenesis – iron scavenging, coagulation and immune evasion – have been extensively studied for vaccine development [33, 34]. In our study we found proteins associated with at least two of

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Table 1
Proteins identified by MALDI-TOF/TOF and predicted/classified as secreted, membrane, cell wall associated proteins or with unknown location.

Spot ID[a]	Protein (abbreviations)	UniProt accession numbers	Protein score[b]	Peptide match (MS)	Peptide match (MS/MS)	*Staphylococcus pseudintermedius* strain	Localization[c,d]	Functional category[c]	Predicted cleavage sites[e]	TMD[f]
Fraction I (S1)										
1248	Uncharacterized protein (LPXTG-motif cell wall anchor domain protein) (SpsN)	E8SI62	520	10	5	HKU10-03	CW	Unknown/unclassified proteins	33–34	2
1465	Immunoglobulin G binding protein A spa1 (SpsQ)	F0P4S0	122	8	1	ED99	CW	Pathogenesis associated proteins	33–34	2
1239	Signal peptidase I (SipB)	E8SEB4	569	9	5	HKU10-03	CW	Protein fate	Predicted as secreted by SecretomeP, TargetP and PrediSi	1
989	Gamma-hemolysin component B	E8SJE6	816	13	8	HKU10-03	S	Pathogenesis associated proteins	26–27	1
1106	Secretory antigen SsaA	E8SEX5	845	6	5	HKU10-03	S	Pathogenesis associated proteins	28–29	2
1674	Immunodominant antigen A (IsaA)	E8SFY1	643	5	5	HKU10-03	S	Pathogenesis associated proteins	29–30	1
452	Lipoteichoic acid synthase LtaS Type Ib	E8SK91	1210	17	11	HKU10-03	M	Cell envelope	Predicted as secreted by SecretomeP, TargetP	5
713	Indole-3-pyruvate decarboxylase (IpdC)	F0P476	1680	19	13	ED99	M	Transport and binding proteins	No	2
713	Pyruvate decarboxylase alpha-keto-acid decarboxylase (Kdc)	E8SFW9	950	18	12	HKU10-03	M	Transport and binding proteins	No	2
684	Alkyl hydroperoxide reductase protein F	E8SI38	485	7	5	HKU10-03	M	Cellular processes	No	2
586	MRP-like scaffold protein for [4Fe–4S] cluster assembly ApbC	E8SE32	205	5	2	HKU10-03	M	Unknown/unclassified proteins	No	1
500	ATP-binding protein, Mrp/Nbp35 family	F0P3H1	293	6	2	ED99	M	Transport and binding proteins	No	1
1654	Iron compound ABC transporter, iron compound-binding protein (IsdE)	F0P3F9	1100	13	11	ED99	M	Transport and binding proteins	31–32	2
713	Signal recognition particle receptor FtsY	E8SG34	231	6	2	HKU10-03	M	Cell envelope	No	0
802	Siderophore staphylobactin ABC transporter, substrate-binding protein SirA	E8SJM0	1050	9	7	HKU10-03	M	Transport and binding proteins	29–30	2
929	Manganese ABC transporter, periplasmic-binding protein SitA	E8SJL4	759	9	9	HKU10-03	M	Transport and binding proteins	23–24	1
1660	Fructose-bisphosphate aldolase class II	E8SDZ4	1110	13	10	HKU10-03	M	Energy metabolism	No	0
1247	Uncharacterized protein (glutamyl-endopeptidase)	E8SIZ1	822	11	8	HKU10-03	M	Protein fate	25–26	2
707	Predicted IgG-binding protein SBI (SpsK)	E8SHU1	635	9	6	HKU10-03	U	Pathogenesis associated proteins	29–30	1
681	Iron–sulfur cluster assembly protein SufD	E8SDS7	910	12	7	HKU10-03	U	Transport and binding proteins	No	0
586	Tellurite resistance protein, putative	F0P6D0	256	12	2	ED99	U	Resistance proteins	Predicted as secreted by SecretomeP	0
1146	Extracellular protein	E8SI37	1070	9	8	HKU10-03	U	Unknown/unclassified protein	22–23	2
1101	Phosphomethylpyrimidine kinase	E8SJF0	453	9	4	HKU10-03	U	Purines, pyrimidines, nucleosides, and nucleotides	No	1
877	Transaldolase	E8SJC3	1170	14	10	HKU10-03	U	Cell envelope	No	0
1174	Uncharacterized protein (glucosamine-6-phosphate isomerase family protein)	E8SJR3	1450	18	11	HKU10-03	U	Unknown/unclassified protein	No	0
929	General stress protein-like protein	E8SJ85	168	4	2	HKU10-03	U	Unknown/unclassified protein	Predicted as secreted by SecretomeP, TargetP and PrediSi	1
1340	Isochorismatase	E8SEG2	669	7	6	HKU10-03	U	Biosynthesis of cofactors, prosthectic groups and carriers	No	0
1428	Signal transduction protein TRAP (target of RNAIII-activating protein)	F0P6T1	604	7	7	ED99	U	Signal transduction	Predicted as secreted by SecretomeP	0
1340	Uncharacterized protein	E8SH76	1330	12	9	HKU10-03	U	Unknown/unclassified protein	Predicted as secreted by SecretomeP	0
1291	Alkaline shock protein 23	E8SE50	785	5	4	HKU10-03	U	Unknown/unclassified protein	Predicted as secreted by SecretomeP	0
1277	Glutathione peroxidase	E8SGL1	859	13	9	HKU10-03	U	Stress response	Predicted as secreted by SecretomeP	0
1461	Uncharacterized protein	F0P4W6	235	5	3	ED99	U	Unknown/unclassified protein	No	0
1370	General stress protein 26	E8SFT5	280	4	4	HKU10-03	U	Unknown/unclassified protein	Predicted as secreted by SecretomeP	0
1682	Uncharacterized protein (putative lipoprotein)	E8SGS1	867	10	7	HKU10-03	U	Unknown/unclassified protein	29–30	1
1186	Peptide methionine sulfoxide reductase MsrB	E8SH38	724	10	7	HKU10-03	U	Transport and binding proteins	Predicted as secreted by SecretomeP	0

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1370	Uncharacterized protein	E8SGZ6	460	4	4	HKU10-03	U	Unknown/unclassified protein	No	0
1500	Uncharacterized protein	E8SE40	962	8	8	HKU10-03	U	Unknown/unclassified protein	Predicted as secreted by SecretomeP	0
Fraction IIa (PFI)										
433	Probable malate:quinone oxidoreductase	E8SGU8	1170	30	11	HKU10-03	CW	Energy metabolism	No	2
982	Beta-hemolysin (Hlb)	F0P8F0	395	9	4	ED99	S	Pathogenesis associated proteins	31–32	1
2328	Synergohymenotropic toxin (LukS)	F0P6U2	759	11	7	ED99	S	Pathogenesis associated proteins	29–30	1
246	Penicillin-binding protein 3 (Pbp3)	F0P4N9	480	20	4	ED99	M	Cell envelope	Predicted as secreted by SecretomeP	1
5063	GTP-binding protein TypA/BipA	E8SF93	551	20	3	HKU10-03	M	Energy metabolism	No	0
5067	ATP synthase subunit beta (AtpD)	E8SKN8	1210	23	11	HKU10-03	M	Energy metabolism	No	0
627	NADH dehydrogenase	E8SE87	715	12	5	HKU10-03	M	Energy metabolism	No	1
5030	Phosphoribosylaminoimidazole carboxylase, ATPase subunit (PurK)	F0P3Y5	383	11	3	ED99	M	Purines, pyrimidines, nucleosides, and nucleotides	No	1
672	NADH dehydrogenase	E8SE84	853	16	6	HKU10-03	M	Energy metabolism	Predicted as secreted by SecretomeP	1
5019	Foldase protein PrsA	E8SJP6	984	13	8	HKU10-03	M	Protein Fate	20–21	1
1087	Enoyl-[acyl-carrier-protein] reductase [NADPH] FabI	E8SEN6	988	12	8	HKU10-03	M	Fatty acid and phospholipid metabolism	No	1
1682	ATP synthase subunit b (AtpF)	E8SKP2	231	4	3	HKU10-03	M	Energy metabolism	Predicted as secreted by TargetP	1
2320	Endonuclease/exonuclease/phosphatase family protein	F0P6K4	1210	31	10	ED99	U	Unknown/unclassified protein	28–29	1
2328	Acid phosphatase	E8SEC6	391	8	5	HKU10-03	U	Protein Fate	23–24	1
1431	Cell division protein sepF	E8SFP6	377	10	2	HKU10-03	U	Cellular processes	No	0
1370	ATP synthase subunit delta	E8SKP1	497	5	4	HKU10-03	U	Transport and binding proteins	No	0
Fraction IIb (PFA)										
108	Zinc metalloproteinase/aureolysin	E8SFX6	143	5	2	HKU10-03	M	Protein fate	26–27	1
82	Lipoprotein	E8SFE0	472	5	3	HKU10-03	S	Unknown/unclassified protein	23–24	1
40	ATP synthase subunit alpha (AtpA)	E8SKP0	249	7	3	HKU10-03	M	Energy metabolism	No	1
53	Do-like Serine protease, DegP/HtrA	E8SJ75	417	8	4	HKU10-03	M	Protein Fate	Predicted as secreted by SecretomeP	1
134	Histidine triad (HIT) nucleotide-binding protein	E8SJP1	392	4	3	HKU10-03	U	DNA metabolism	No	0
Fraction IIIa (S2FI)										
639	Thiol peroxidase, Bcp-type	E8SJS3	274	4	3	HKU10-03	CW	Cellular processes	No	0
76	N-acetylmuramoyl-L-alanine amidase, family 4	E8SGA9	246	6	3	HKU10-03	S	Cell envelope	27–28	1
407	Zinc-binding lipoprotein, putative	F0P366	220	19	2	ED99	M	Transport and binding proteins	28–29	1
401	Iron–sulfur cluster assembly protein SufB	E8SDT0	507	13	5	HKU10-03	U	Transport and binding proteins	No	1
473	Malate dehydrogenase	E8SJY3	245	5	2	HKU10-03	U	Energy metabolism	No	2
576	D-Alanine aminotransferase	E8SJ96	768	13	8	HKU10-03	U	Amino acid biosynthesis	No	0
703	UPF0173 metal-dependent hydrolase	F0P3J9	385	5	4	ED99	U	Transport and binding proteins	No	0
720	Deoxyadenosine kinase/Deoxyguanosine kinase	E8SJ42	483	6	5	HKU10-03	U	Purines, pyrimidines, nucleosides, and nucleotides	Predicted as secreted by SecretomeP	0
811	Probable thiol peroxidase	F0P3J2	894	8	6	ED99	U	Cellular processes	No	0
750	Putative tRNA (cytidine(34)-2′-O)-methyltransferase	E8SJR4	493	5	4	HKU10-03	U	Transcription	Predicted as secreted by SecretomeP	0
863	N5-carboxyaminoimidazole ribonucleotide mutase	E8SF47	301	4	3	HKU10-03	U	Purines, pyrimidines, nucleosides, and nucleotides	No	1
819	Staphylococcal accessory regulator family protein	E8SGM7	410	9	5	HKU10-03	U	Regulatory functions	No	0
Fraction IIIb (S2FA)										
9	Fibronectin binding protein FnbB (SpsD)	E8SH04	753	11	8	HKU10-03	CW	Pathogenesis associated proteins	36–37	2
76	Superoxide dismutase SodA	E8SI20	939	12	9	HKU10-03	S	Cellular processes	Predicted as secreted by SecretomeP	0
55	Uncharacterized protein (putative lipoprotein)	E8SF37	347	6	3	HKU10-03	U	Unknown/unclassified protein	17–18	1
77	Uncharacterized protein	E8SGS5	401	7	6	HKU10-03	U	Unknown/unclassified protein	Predicted as secreted by SecretomeP	0

[a] Spots ID can be observed in Supplementary Fig. 1.
[b] Identification score obtained with the Mowse algorithm. A result was considered to be significant when a score above 70 was attained.
[c] Predicted by PSORTb v3.0 trained on Gram-positive (CW, cell wall; M, membrane; S, secreted; U, unknown).
[d] Functional role category assignments for each of the 361 unique proteins based on Gillaspy et al. [18] and Blast2go.
[e] Predicted by SignalP v4.1, and one of the following algorithms SecretomeP v2.0, TargetP v1.1 and/or PrediSi. The numbers in this column represent the predicted cleavage site by SignalP v4.1 (e.g. 28–29 corresponds to a cleavage site between positions 28 and 29).
[f] Number of transmembrane domain (TMD) as predicted by TMpred.

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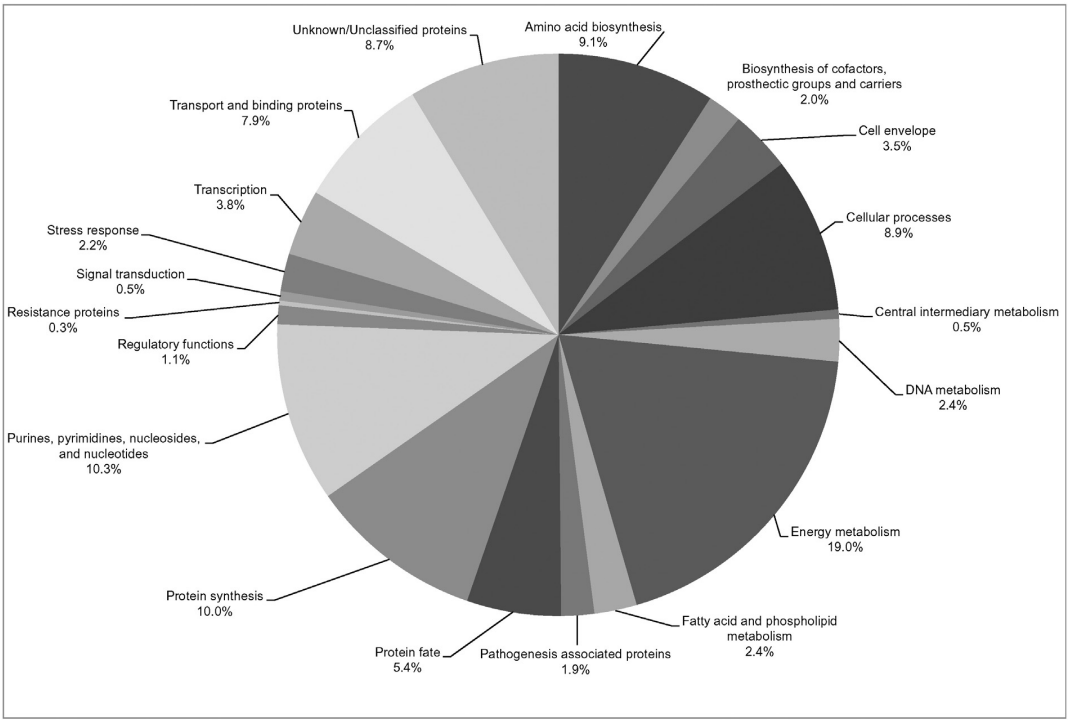

Fig. 3. Functional role category assignments for each of the 361 unique proteins based on Gillaspy et al. [18] and Blast2go.

these areas: iron scavenging — Iron compound ABC transporter (IsdE) and Siderophore staphylobactin ABC transporter (SirA) and immune evasion — IgG-binding protein SBI (SpsK) and Immunoglobulin G binding protein A spa1 (SpsQ). IsdE is a protein of the iron-regulated surface determinant (Isd) pathway, which represents the main heme transporter of *S. aureus* [33]. Interestingly IsdE and SirA of *S. aureus* are lipoproteins strongly immunogenic, which induce an inflammatory response through recognition by Toll-like receptor (TLR) 2-MyD88 [35]. Since iron-acquisition is essential for staphylococcal survival and these lipoproteins are highly immunogenic, it seems like they are a good choice of targets for vaccine development. Moreover targeting multiple iron uptake systems seems to be a good formulation to maximize efficacy. This is due to the plasticity of staphylococci, meaning they have the ability to remain pathogenic when only limited antigens are neutralized because they have so many virulence factors, including iron uptake systems [36].

SpsK and SpsQ are two immunoglobulin-binding molecules, which prevent opsonization [33]. In *S. aureus* these proteins' homologues, Sbi and Spa respectively, severely limit host immunoglobulin mediated immune clearance of the pathogen [33]. Despite being very abundant staphylococcal surface proteins for potential interaction with the host immune surveillance system, these proteins are not good candidates for a vaccine due to its toxicity and B cell super-antigen activity [33]. However, slightly modified proteins could be of potential use without its deleterious effects.

Vaccines composed of single or multiple antigens are more favorable compared to attenuated or killed whole bacteria vaccines, since many components of staphylococci may cause adverse effects [12]. Usually surface proteins have higher probability of interaction with host's immune system and therefore are usually chosen for the purpose of vaccine development. Using the proteome characterization approach we identified several proteins (Table 1), which could be potentially included in a multivalent vaccine against *S. pseudintermedius*. We then used the SERPA approach to further select potential vaccine candidate antigens.

3.3. Characterization of highly immunogenic proteins identified by SERPA

In order to assess the immunogenic potential of the previously identified proteins, a Western blot analysis of the two-dimensional gels was carried out with serum from healthy dogs, dogs with atopic dermatitis infected and not infected with *S. pseudintermedius*. As expected, the dog's serum from which *S. pseudintermedius* strain FMV5819/10 was isolated was the one that recognized more proteins. Several highly immunogenic areas (containing more than one spot) were detected in the cell wall fraction (Fig. 4a), and in the membrane associated fraction (Fig. 4b). Table 2 shows which immunogenic areas each type of serum identified. For the cell wall fraction areas A, C, D and E were identified by more than 50% of the ADI sera and less than 50% of the H sera. In the membrane fraction only areas C, E and F met the established criteria. *S. pseudintermedius* is an opportunistic pathogen and it resides as part of the normal flora of most dogs and does not cause any disease unless the resistance of the host is lowered and the skin barrier altered by predisposing factors, such as atopic dermatitis [37]. However, not all dogs with atopic dermatitis develop infection by *S. pseudintermedius* and remain solely colonized by this bacterium. We used the sera from the ADH group, in an attempt to identify antigenic proteins for which specific antibodies were missing or underrepresented in infected patients that could contribute to prevention of infection, however we could not detect spots more associated with these non-infected dogs than with infected dogs. This suggests the anti-staphylococcal antibody repertoires from patients with atopic dermatitis with or without *S. pseudintermedius* pyoderma overlap, meaning that in dogs with atopic dermatitis, *S. pseudintermedius* expresses similar proteins during invasive disease and during colonization.

For the purpose of knowing if the proteins identified could potentially induce an undesirable IgE response, we also identified highly immunogenic areas after incubation with anti-dog-IgE peroxidase conjugate. The IgE immunogenic areas are shown in Fig. 5. These areas corresponded to area B in the cell wall associated fraction and areas C, D and E in the

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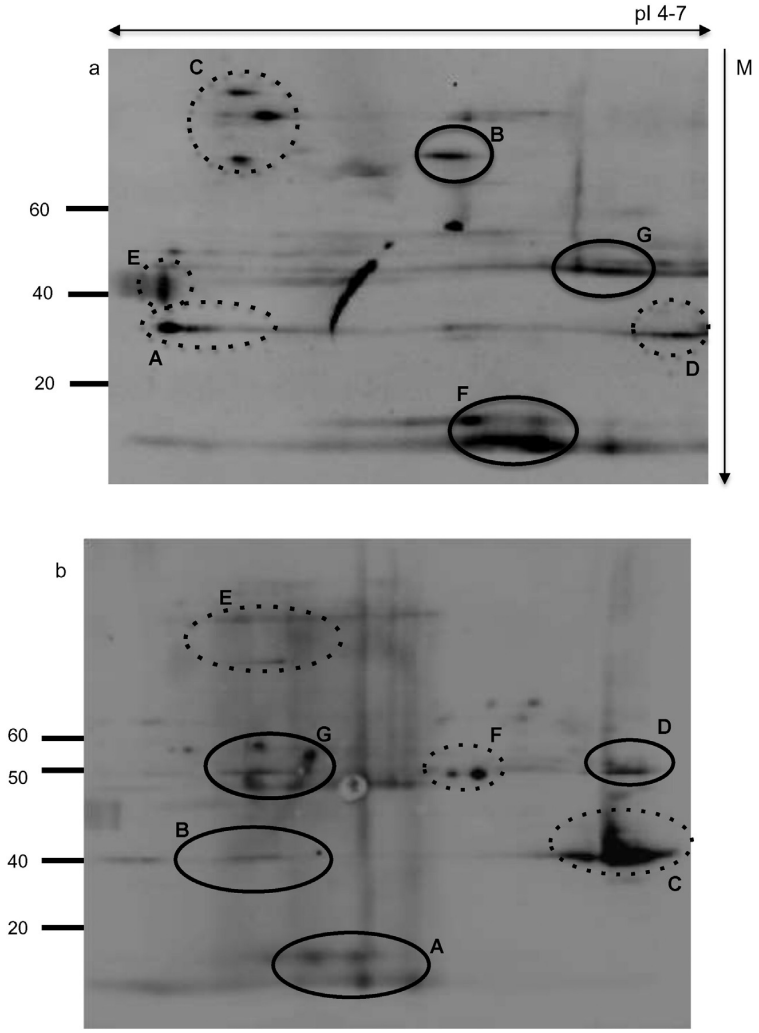

Fig. 4. Representative SERPA of cell wall associated fraction (a) and membrane fraction (b) from *S. pseudintermedius* FMV5819/10 using the dog's serum from which the strain was collected (dog with atopic dermatitis and pyoderma by *S. pseudintermedius*) and the anti-dog-IgG peroxidase conjugate. The highly immunogenic areas are represented inside the circles. Dotted circles were further studied since they met the criteria of areas identified by ≥50% of the ADI sera and identified by <50% of the H sera.

Table 2
Number of sera in each group that detected the different highly immunogenic areas of *S. pseudintermedius* FMV5819/10.

Immunogenic area	Number of sera (%)		
	Healthy dogs (n = 13)	Dogs with atopic dermatitis with recurrent *S. pseudintermedius* infections (n = 17)	Dogs with atopic dermatitis without infections (n = 8)
Cell wall fraction			
A	**6 (46%)**	**17 (100%)**	**6 (75%)**
B	10 (77%)	16 (94%)	8 (100%)
C	**6 (46%)**	**16 (94%)**	**6 (75%)**
D	**2 (9%)**	**13 (76%)**	**5 (63%)**
E	**3 (23%)**	**9 (53%)**	**2 (25%)**
F	0 (0%)	4 (24%)	0 (0%)
G	0 (0%)	5 (29%)	2 (25%)
Membrane fraction			
A	6 (50%)	12 (71%)	5 (63%)
B	6 (50%)	13 (76%)	5 (63%)
C	**3 (23%)**	**9 (88%)**	**6 (75%)**
D	3 (23%)	6 (35%)	1 (13%)
E	**2 (9%)**	**13 (76%)**	**3 (38%)**
F	**1 (8%)**	**8 (50%)**	**3 (38%)**
G	4 (31%)	6 (35%)	1 (13%)

Areas in bold were further studied since they met the criteria of areas identified by ≥50% of the ADI sera and identified by <50% of the H sera.

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Table 3
Highly immunogenic antigens from *S. pseudintermedius* FMV5819/10 strain.

Immunogenic areas[a]	Protein (abbreviations)	Subcellular localization[b]	Functional category[c]
Cell wall fraction			
A1	50S ribosomal protein L9	Ribosome	Protein synthesis
A2	30S ribosomal protein S7	Ribosome	Protein synthesis
C1	**Fibronectin-binding protein SpsD**	**Cell wall**	**Pathogenesis associated proteins**
C2	Trigger factor Tig	Cytoplasm	Cellular processes
C3	Chaperone protein DnaK	Cytoplasm	Protein Fate
C4	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	Cytoplasm	Energy metabolism
C5	60 kDa chaperonin GroL	Cytoplasm	Protein Fate
C6	Phosphoglycerate kinase Pgk	Cytoplasm	Energy metabolism
D1	**Lipoprotein, putative**	**Unknown**	**Unknown**
E1	50S ribosomal protein L3	Ribosome	Protein synthesis
E2	50S ribosomal protein L5	Ribosome	Protein synthesis
Membrane fraction			
F1	**ATP synthase subunit alpha AtpA**	**Membrane**	**Energy metabolism**
F2	**Enoyl-[acyl-carrier-protein] reductase [NADPH] FabI**	**Membrane**	**Fatty acid and phospholipid metabolism**

Proteins marked in bold were those considered with the highest potential as vaccine candidate antigens.
[a] Immunogenic areas identified by Western blot analysis according to the criteria of areas identified by ≥50% of the ADI sera and identified by <50% of the H sera. Immunogenic areas identified by IgE-containing serum were not included in this table.
[b] Predicted by PSORTb v3.0 trained on Gram-positive.
[c] Functional role category assignments for each protein based on Gillaspy et al. and Blast2go.

lipoproteins are a major class of cell surface-exposed proteins in many bacterial pathogens and play critical roles in nutrient uptake, antibiotic resistance, adhesion, protein secretion, and other functions [40]. These molecules have been the targets of vaccine research for many pathogenic bacteria. A putative lipoprotein of *S. aureus* was identified as highly immunogenic [12]. However, much of these lipoproteins, as in the case of our lipoprotein, do not have an annotated function and we cannot anticipate the result of blocking this protein in vivo.

AtpA participates in the synthesis of ATP by driving the flow of protons into the cell, generating a proton motive force, which energizes processes such as motility and active transport [41]. In *Salmonella* Typhimurium, an *atpA* deletion was responsible for the attenuation of the bacterial in vivo growth and furthermore was found to offer significant protection against subsequent challenge [41]. These mutants were attenuated for virulence and effectively protected mice against *Salmonella* infection [41]. In staphylococcal species, one study showed that knocking down the ATP synthase expression strongly suppressed the growth of *S. aureus*, revealing a crucial role of this target in bacterial growth and metabolism [42]. These studies together really suggest that ATP synthase proteins may be good targets of vaccines against staphylococci.

The Enoyl-[acyl-carrier-protein] reductase [NADPH] FabI protein has been described as essential for the *S. aureus* fatty acid biosynthesis and, hence, serves as an attractive drug target [43]. In fact there are several compounds that successfully inhibit FabI, such as the first-line tuberculosis pro-drug isoniazid and the FabI diphenyl ether inhibitor triclosan, which is recommended as topical antiseptic to reduce methicillin-resistant *S. aureus* skin colonization [43]. Combined with the fact that this protein is located in the cellular membrane makes FabI an approachable target candidate.

By using whole proteome characterization or serological proteomic analyses we were able to identify 39 and 13 proteins with potential to be used as vaccine candidates, respectively. Interestingly the 4 proteins considered as highly promising candidate therapeutic targets were identified by both strategies.

In this approach we were able to identify several new antigens with potential for vaccine development. Further studies are now needed to evaluate the presence of these proteins in other *S. pseudintermedius* strains and so determine their frequency in clinical isolates. This will allow determining a combination of the appropriate antigens as a potential vaccine against *S. pseudintermedius*.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.12.017.

Conflicts of interest

The authors have no conflict of interest to declare.

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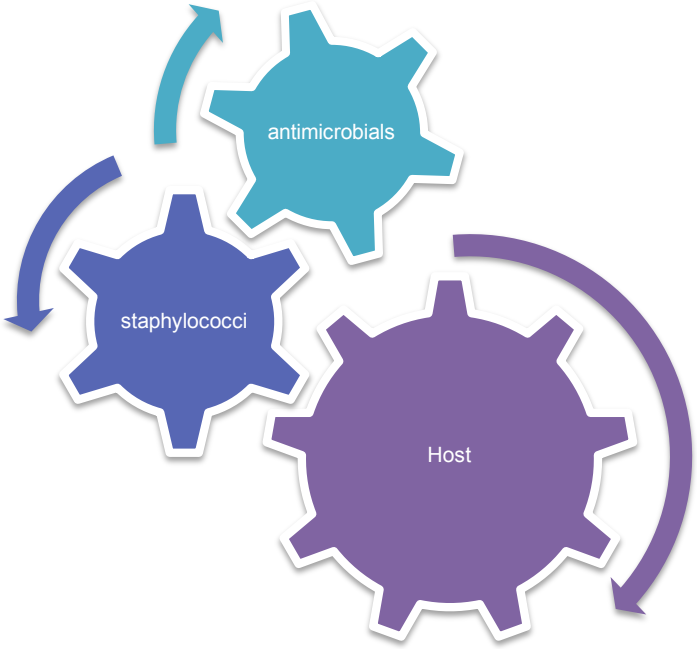


4 Discussion

Staphylococci are a group of bacteria with clinical, agricultural, and economic importance because of their wide range of virulence factors and ability to become resistant to antimicrobials. Monitoring programs may help uncover new resistance trends and evaluate the usefulness of the available antimicrobials, in veterinary medicine, against staphylococci.
In particular, the recent emergence of MRSP has complicated considerably the treatment of infections caused by these bacteria (Frank & Loeffler, 2012). MRSP have become virtually resistant to all the antimicrobials approved for administration in companion animals, which has led to ethical concerns about the use of antimicrobials classified by the World Health Organization as “critically important” for human medicine (Frank & Loeffler, 2012).

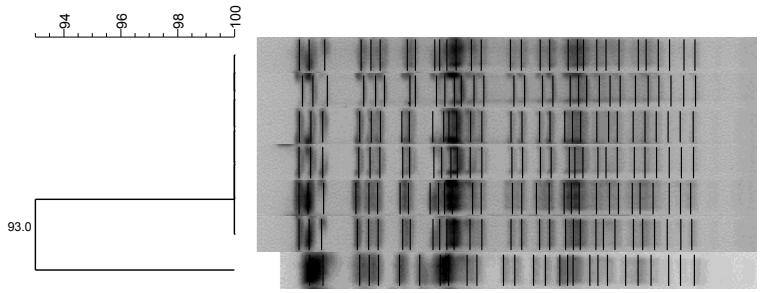
4.1 MRSA in animals, environment and humans in close contact with animals

MRSA is of significant concern in human and veterinary medicine (Weese and van Duijkeren, 2010). The aim of this part of the study was to get information on the frequency and characteristics of MRSA from colonization and infection samples. Previous studies had been conducted in pigs and companion animals in Portugal (Pomba et al., 2009; Pomba et al., 2010a; Couto et al., 2011) and so a primary focus on studies in calves and horses was performed. The frequency of MRSA nasal colonization was 2% in calves and 3% in horses. The frequency found in horses was within the prevalence rate found in other studies, which range between 0% and 12% depending on the country of isolation (Weese and van Duijkeren, 2010). Interestingly the two MRSA strains from horses were from two different lineages: one belonged to ST398, which is the predominant clone among horses in Europe (Weese and van Duijkeren, 2010) and the other was ST5, a clone that has only been identified in horses sporadically (Vincze, Stamm, Kopp, Hermes, Adlhoch, Semmler, Wieler, Lubke-Becker & Walther, 2014). Both strains were multidrug-resistant and carried several antimicrobial resistance genes.
On the other hand, the six MRSA isolates obtained from calves were all ST398 and were isolated from only one of the two farms studied (Farm A). The six strains were also resistant to fluoroquinolones, tetracyclines and phenicols. This farm was using enrofloxacin routinely to avoid respiratory and/or gastrointestinal infections. Previous administration of fluoroquinolones is an identified risk factor for MRSA infection in companion animals (Weese and van Duijkeren, 2010). So, the use of enrofloxacin in Farm A could have imposed a selective pressure that led to the spread of the MRSA strains found. Farm B, on the other hand, was using gamithromycin for the same purpose and it seems this antimicrobial did not select for MRSA. The *mecC* gene was not detected in any *S. aureus* strains, which was unexpected since *mecC*-carrying MRSA have been detected in several cows throughout Europe (Garcia-Alvarez et al., 2011; Becker et al., 2013; Loncaric et al., 2013).

Although the frequency of MRSA colonization was low in both species, horses and calves can still be considered important reservoirs of MRSA strains, which can be disseminated from these animals to the environment, to other animals and eventually to humans.

After the first two studies on colonization in horses and calves, the next step was to characterize all the MRSA isolated at the Antibiotic Resistance Laboratory from 2001 to 2014. These included strains from animals, humans in close contact with animals and environmental. Overall, fourteen *spa* types were identified and according to the *spa* server, five of these were associated with CC398 (n=47); six were hinted to CC22 (n=21) and three were associated with CC5 (n=6). All MRSA from livestock-animals (pigs and calves) were CC398 and most strains from companion animals (dogs, cats and horses) were CC5 and CC22. The majority of the isolates (74%) were multidrug-resistant. One MRSA ST398 strain was also isolated from a clinical sample from one dog. This MRSA ST398 strain had 93% *Apa*I-PFGE similarity to the MRSA CC398 strains from calves (Figure 13).

Figure 13. Dendogram of chromosomal DNA digested with *Apa*I of MRSA ST398 strains from six calves and one dog.



Species	*spa* type	MLST	Source
Bovine	t108	CC398	Nasal swab
Bovine	t108	CC398	Nasal swab
Bovine	t108	CC398	Nasal swab
Bovine	t108	CC398	Nasal swab
Bovine	t108	CC398	Nasal swab
Bovine	t108	CC398	Nasal swab
Dog	t108	ST398	Skin

Surprisingly all these strains (from the dog and calves) carried the *fexA* gene and were resistant to fluoroquinolones, which suggests they had a similar source of infection/colonization. However, the dog had no history of contact with farms or farm animals and so the route of transmission remains to be elucidated.

As expected, all CC22 strains were SCC*mec* IV. The ST22-IV was the most common MRSA lineage found in companion animals. This is in agreement with previous reports, which show that there is a shared population of this lineage infecting/colonizing humans and companion animals (Harrison et al., 2014). The ST22-t032-SCC*mec* IV strains were negative for the *erm*(C) gene, while the other ST22-IV non-t032 (t2357 and t025) strains carried this gene. It is assumed that the loss of this gene occurs in companion animals (Harrison et al., 2014), and this suggests that the MRSA ST22 non-t032 strains found in our study were acquired from humans very recently, and they have thus maintained the *erm*(C) gene.

The other MRSA strains isolated from companion animals belonged to CC5 (ST5 and ST105), the second human epidemic MRSA clone most commonly isolated in Portugal (Espadinha et al., 2013). This suggests that CC5 is spread in the animal population, and in humans both in the hospital and community settings in Portugal (Espadinha et al., 2013). The

first Portuguese, and also European, VRSA human strain isolated in 2013 was ST105-II (Melo-Cristino et al., 2013; Friães, Resina, Manuel, Lito, Ramirez & Melo-Cristino, 2014). VRSA isolates from other countries also belonged to CC5 (Friães et al., 2014). In addition to the possibility of companion animals being a reservoir and distributer of VRSA, companion animals can also carry *vanA*-carrying vancomycin-resistant enterococci (Poeta et al., 2005) and thereby raise the chance for the acquisition of the *vanA* gene by staphylococci.

Pets-associated (PA)-MRSA (CC5 and CC22) were significantly more likely to carry enterotoxin genes (*seg* [p=0.002], *sei* [p=0.002], *sem* [p=0.002], *sen* [p=0.002], *seo* [p=0.002], *seu* [p=0.002], *egc* [p=0.002]) and staphylokinase gene (*sak* [p=0.015]) than MRSA CC398. This supports the previous observation that companion animals, share a population of an important and globally disseminated lineages of MRSA, namely ST22-IV that can infect both humans and other companion animals without undergoing host adaptation (Harrison et al., 2014). Companion animals seem to carry *S. aureus* clonal linages more virulent to humans then livestock-animals and so active surveillance including companion animals seems urgently needed.

4.2 Epidemiology, antimicrobial and biocide susceptibility of staphylococci isolated from animals in Portugal

Knowledge on the antimicrobial and biocide susceptibility and molecular epidemiology of MRS strains, other than MRSA, is important to monitor the spread of antimicrobial/biocide resistance genes/strains and consequentely control its dissemination.

4.2.1 Antimicrobial susceptibility

The staphylococci isolated from livestock-animals in this study were almost all multidrug-resistant. In fact, MRSA CC398 were significantly more likely to carry particular antimicrobial resistance genes (*dfrK* [p=0.0001], *tet*(K) [p=0.0001], *tet*(M), [p=0.0001], *vga*(A) [p=0.0001]), when compared to the other MRSA clonal complexes (CC5 and CC22). The high selective pressure imposed by the consumption of antimicrobials in food-producing animals may lead to the acquisition of these genes by MRSA CC398. Furthermore these antimicrobial resistance genes are usually carried in the same mobile genetic elements (Wendlandt, Shen, Kadlec, Wang, Li, Zhang, Feßler, Wu & Schwarz, 2015) and so are co-selected by the use of differente antimicrobials, aiding to the persistence of MRSA in the environment.

The MRS isolates from horses were resistant to one other antimicrobial (mainly tiamulin and tetracycline) besides ß-lactams, although the corresponding mechanisms of resistance were not always identified. In staphylococci isolated from companion animals, there were increasing resistance trends for ampicillin, penicillin, amoxicillin/clavulanic acid, cefoxitin in *S. aureus* and CoNS, oxacillin in *S. pseudintermedius*, cefovecin, cephalexin, cefotaxime, ceftriaxone, ciprofloxacin, enrofloxacin, norfloxacin, ofloxacin, moxifloxacin, tetracycline, chloram-

phenicol, gentamicin, neomycin, tobramycin, kanamycin, streptomycin, erythromycin, clindamycin, sulfamethoxazole/trimethoprim sulphonamides and trimethoprim. Moreover, increasing trends of resistance to at least one antimicrobial and multidrug-resistance were also identified, such that almost 35% of the staphylococci strains were multidrug-resistant. All MRSP and several MRCoNS strains were multidrug-resistant. The finding of multidrug-resistant MRSP and MRCoNS strains in Portugal is not surprising since there have been several reports describing this observation (Schnellmann et al., 2006; Fitzgerald, 2009; Weese and van Duijekeren, 2010; Frank & Loeffler, 2012). However, some methicillin-susceptible strains also exhibited a multidrug-resistant profile, suggesting a spread of antimicrobial resistance genes in the animal staphylococcal population.

An important finding was the detection of the *fexA* gene, conferring resistance to florfenicol and chloramphenicol, in 2 *S. pseudintermedius* strains. In *S. pseudintermedius*, only a *fexA* variant named *fexAv*, had been previously reported and conferred resistance only to chloramphenicol (Gómez-Sanz et al., 2013). The animals infected with these strains were being treated with florfenicol (25-50mg/kg q12h SC, Nuflor®, Merck Animal Health, USA) because they had been previously diagnosed with an infection caused by a multidrug-resistant MRSP strain and so florfenicol was being used as a last-resort antimicrobial. The use of florfenicol was very recently suggested, as a second-line antimicrobial agent in dogs (Maaland et al., 2015). However, it seems that the use of this antimicrobial can lead to the acquisition of antimicrobial resistance genes or strains. Furthermore, one of these strains also carried the *cfr* gene. This is the first description the *cfr* gene in a *S. pseudintermedius* strain. Although the strain did not exhibit resistance to linezolid, this is a worrisome finding since it shows *S. pseudintermedius* could be carriers of important resistant genes. Although direct selective pressure by the use of the linezolid was not applied, it seems florfenicol may co-select for linezolid-resistance genes due to co-location in the same mobile genetic elements. So the use of florfenicol as a second-line antimicrobial agent in dogs should be reconsidered.

4.2.2 Biocide susceptibility

Considering biocides, several susceptibility patterns and mechanisms of biocide tolerance were found in different staphylococcal species. In fact, numerous efflux pumps have been recognized in staphylococci, but QAC efflux proteins seem to be the most widespread (Bjorland et al., 2003; Costa et al., 2013). In this study, the *qacG* and *qacJ* genes were described for the first time in MRSA CC398 strains. The *qacG* gene had been described in porcine MRSA isolates from clonal lineage ST9 in Hong Kong (Wong, Zhang, O'Donoghue & Boost, 2013) and both genes have also been detected among staphylococci of bovine and caprine origin in Norway (Bjorland et al., 2005). Biocides are extensively used in animal husbandry, including quaternary ammonium compounds (Bjorland et al., 2005). The acquisition of these efflux-pump genes by MRSA CC398, mainly carried on plasmids, may aid to the persistence

of MRSA in the environment, making the eradication of MRSA CC398 more difficult. Two MRS strains from horses, one *S. haemolyticus* and one *S. cohnii* subsp. *cohnii* also carried plasmid-borne *qac* genes: a *qacA* and *sh-fabI* in *S. haemolyticus* and *qacB* and a *qacH*-like in *S. cohnii* subsp. *cohnii*. However, none of the two *qac*-positive strains had high MBC values to benzalkonium chloride, chlorhexidine or gluteraldehyde. Indeed, all MBC values were lower than the recommended in-use concentrations for veterinary medicine (SCENIHR, 2009). This means that biocides are still a reliable antiseptic option against MRS in horses. Nevertheless, when comparing the MBC values of the isolates of the present study with the concentration of BAC in human products (100mg/mL) (Narui et al., 2007), five isolates with benzalkonium chloride MBCs of >128mg/mL were detected. This is of notice, since transmission of MRS strains between horses, humans and their environments may occur (Weese and van Duijkeren, 2010). Similarly, one MSSP isolate harboured a *qacA* gene, while in another a *qacB* gene was detected. Three of the biocide products studied had high bactericidal activity, even against the *qac*-positive strains (Otodine®, Clorexyderm Spot Gel®, Dermocanis Piocure-M®), although Skingel® failed to achieve a five log reduction in the bacterial counting. *S. pseudintermedius* has become a serious therapeutic challenge in particular if methicillin-resistance and/or multidrug-resistance are involved, yet biocides, like chlorhexidine acetate and triclosan, seem to be clinically effective and safe topical therapeutic options against *S. pseudintermedius*, including MRSP. Finally, four MRSA strains (three CC5 and one CC22) did not carry *qac* genes but instead had insertion of sequences CAAT (n=3) or GTTGTAATACAAT (n=1) in the -10 motif of the *norA* promoter. These insertions could induce a higher expression of the NorA efflux pump, which could lead to extrusion of certain biocides, namely benzalkonium chloride and also low-levels of fluoroquinolones (Yoshida et al., 1990).

4.2.3 Epidemiology of MRSP

The first MRSP strain appeared in 2007 in Portugal, but only in 2010 we observed an increase in the number of strains. The first MRSP strains in Europe were detected in 2005 (Perreten et al., 2010), and were ST71-II-III. Interestingly, our first MRSP strain isolated in 2007 was ST196-V, and only in 2009 the first ST71-II-III appeared. Between 2009 and mid-2012, MRSP CC71-II-III was the only lineage detected. Yet, in 2013-2014 we observed a higher genetic diversity among the MRSP strains isolated, with other MRSP lineages appearing and actually a new ST, ST400, with the *mecA* gene. The ST45-nt, ST339-III and ST342-IV lineages were already described in recent studies (Perreten et al., 2013; McCarthy et al., 2014). ST45 was the predominant MRSP clonal lineage in Thailand and Israel, and was not typeable by SmaI-PFGE and SCC*mec* typing (Perreten et al., 2013). This lineage carried a novel pseudo-SCC*mec* element, ΨSCC*mec*$_{57395}$ that besides *mecA* also carried determinants of resistance to heavy metals, such as arsenic, cadmium, and copper (Perreten et al.,

2013). It seems that this ST has also been introduced in Europe (according to the MLST database ST45 has been detected in England, The Netherlands and now Portugal). The new ST, ST400 does not belong to any of the previous *mecA*-positive clonal complexes, and it suggests that this ST has acquired SCC*mec*. Two of the MRSP ST400 strains were isolated from two dogs that lived in the same kennel. However, the third dog had no connection to these dogs or to the kennel, which could mean this lineage is already spreading through the dogs' population. *S. pseudintermedius* have become a serious therapeutic challenge and new MRSP lineages are emerging in several countries, including Portugal.

4.2.4 Epidemiology of MRCoNS

All *S. sciuri* isolates from horses showed indistinguishable *Sma*I PFGE profiles, although the isolates were from individual horses, except for two strains, which were isolated from the same horse. These two isolates differed, however, in their tetracycline resistance pheno- and genotype. This observation suggests that this clone of *S. sciuri* is widely disseminated among the horse population in Portugal.

The MRSE STs found in companion animals were identical to the ones previously isolated in humans (community- and hospital-acquired isolates) in Portugal (Rolo et al., 2012a). This means MRSE strains can also circulate in animals, making them a reservoir of important MRSE lineages. Unfortunately there is no MLST database for *S. haemolyticus*, which makes it impossible to compare our strains to other animal or even human strains. Either way, it is important to notice that MRCoNS were more frequently isolated then predicted and the presence of the *mecA* gene was highly associated with these strains. Furthermore several MRCoNS exhibited a multidrug-resistance pattern, suggesting they are reservoirs of antimicrobial resistance genes. So, there is a possibility that MRCoNS are being neglected as important sources of antimicrobial resistant strains/genes.

4.3 Pathogenesis of *S. pseudintermedius* infections in dogs

This dissemination was incredibly rapid but the reasons for the fast emergence and success of these lineages are not fully understood (Latronico, Moodley, Nielsen & Guardabassi, 2014). The "omics" era (genomics, transcriptomics and proteomics) has brought new tools that provide information about cell's genetic background, their potential regulatory mechanisms and biological activity of virulence determinants (Adamczyk-Poplawska, Markowicz & Jagusztyn-Krynicka, 2011). Genomic and proteomic studies, conducted in the last few years, are giving the first clues on the pathways by which MRSP have become successful (McCarthy et al., 2015). In this study a transcriptome analysis of the *in vitro* transcriptional profiles of a clinical MRSP isolate (ST71) and a clinical MSSP isolate (ST379) was done using RNA-seq. Several genes encoding virulence factors were highly expressed in the MRSP strain: *spsA*, *spsB*, *spsD*, *spsK*, *spsL*, *spsN*, gamma-hemolysin component B gene (*hlgB*), both

subunits of *luk-I* gene (*lukF-I* and *lukS-I*), the coagulase and thermonuclease genes (*coa* and *nucC*, respectively). The higher expression of genes encoding proteins able to adhere to the extracellular matrix, like for example *spsD* (encoding a fibronectin-binding protein) and *spsL* (encoding another fibronectin-binding protein) might explain the previous observation that MRSP ST71 strains have higher adherence to corneocytes than non-ST71 strains (Latronico et al., 2014). The differences observed in the expression of virulence factors could be explained by the different expression of virulence regulatory genes between the 2 strains, with *agr* highly expressed in MSSP and *saeRS* in MRSP. Differences in the expression of regulatory genes have been detected in two MRSA clonal lineages, USA300 (ST8) and USA400 (ST1), which have different virulence characteristics (Jones, Montgomery, Boyle-Vavra, Shatzkes, Maybank, Frank, Peterson & Daum, 2014). In fact, USA300 has been considered hypervirulent, compared with lineage USA400, however, USA300 does not contain much more virulence genes than USA400, but it does have an alteration in the expression of regulatory genes and an increased expression of toxins (Jones et al., 2014). Furthermore, the lower expression of the subunit R in the MRSP strain could also suggest that MRSP are more efficient in acquiring mobile genetic elements than MSSP. Indeed it has been shown that MRSP genomes carry more prophages than MSSP strains (McCarthy et al., 2015). Besides carrying more prophages, the results found in this study suggest the MRSP strain also upregulates several phage-associated genes, which could be linked to the upregulation of the integrase and other genes with unknown function (might encode not yet known virulence factors) located in the superantigen-encoding pathogenicity islands (SaPIs). The upregulation of prophage particles is also concordant with the suggestion that transfer in MRSP is predominantly made by bacteriophage transduction (McCarthy et al., 2015). Overall, MRSP isolates seem to highly express a number of virulence genes that could explain its high and fast adaptation.

Since the emergence of MRSP strains, virtually resistant to all the antimicrobials approved for administration in companion animals (Frank & Loeffler, 2012), researchers have focused on the development of new treatment strategies, namely vaccines (Fitzgerald, 2009). One way of identifying potential vaccine candidates is through proteomics (Adamczyk-Poplawska et al., 2011). Proteomics has the advantage of dealing directly with proteins, which are the main players of life processes (Hecker, Becher, Fuchs & Engelmann, 2010). Proteome characterization of bacterial species, especially of their surface-exposed protein fractions, can lead to the identification of vaccine candidates, which can then be tested for their ability to induce protective immunity or as passive immunization targets (Adamczyk-Poplawska et al., 2011). This can be accomplished *in silico*, using bioinformatics tools, and/or by combining proteomics with serological analysis (SERPA – SERological Proteome Analysis) (Movahedi & Hampson, 2008). This approach has enabled the identification of vaccine candidates against *S. aureus* (Vytvytska et al., 2002). Usually surface exposed proteins are preferred for

vaccine development since these proteins are more likely to interact with the host immune system (Fitzgerald, 2009). Moreover, to design potent and generally applicable subunit vaccines, it is necessary to identify those antigens that are recognizable on a wide patient population during infection (Etz et al., 2002). For this reason, serum from dogs with atopic dermatitis and recurrently infected with *S. pseudintermedius* (ADI) was used to detect proteins only expressed during infection and the proteins identified by healthy dogs (H) were discarded. A third group was used, dogs with atopic dermatitis and not recurrently infected with *S. pseudintermedius* (ADH), in an attempt to identify antigenic proteins for which specific antibodies were missing or underrepresented in infected patients that could contribute to infection prevention. There was a clear difference in the immune response of healthy dogs and dogs with atopic dermatitis with or without *S. pseudintermedius* recurrent infections. However, the immune reponse of dogs with atopic dermatitis either with *S. pseudintermedius* recurrent infections or without was the same and was independent from the colonization/infection status (ADI versus ADH). No protective proteins were identified in dogs with atopic dermatitis but without *S. pseudintermedius* recurrent infections (ADH) that could potentially induce an IgG-mediated response against *S. pseudintermedius* infection.

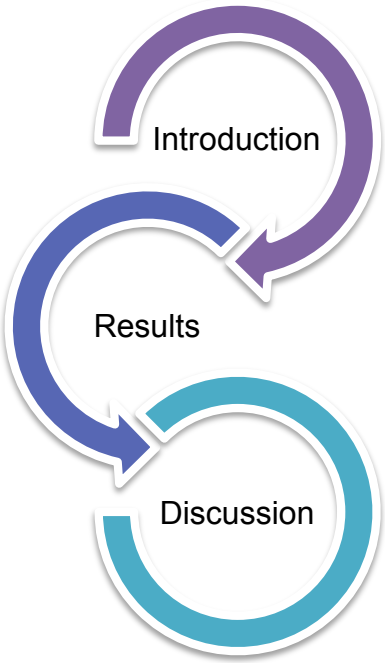
By proteomics, 361 unique proteins were identified, of which 39 were surface-located. These included proteins that have been extensively studied for vaccine development in *S. aureus* (DeDent, Kim, Missiakas & Schneewind, 2012): iron scavenging - Iron compound ABC transporter (IsdE) and Siderophore staphylobactin ABC transporter (SirA); and immune evasion - IgG-binding protein SBI (SpsK) and Immunoglobulin G binding protein A spa1 (SpsQ). SpsK and SpsQ are two immunoglobulin-binding molecules, which prevent opsonisation (DeDent et al., 2012). In *S. aureus* these proteins' homologues, Sbi and Spa respectively, severely limit host immunoglobulin mediated immune clearance of the pathogen (DeDent et al., 2012). Despite these proteins abundancy, they are not good candidates for a vaccine due to its toxicity and B cell super-antigen activity unless slight modifications are introduced in the proteins that could be of potential use without its deleterious effects (DeDent et al., 2012).

In order to assess the immunogenic potential of the previously identified proteins, a Western blot analysis of the two-dimensional gels was carried out with serum from healthy dogs, dogs with atopic dermatitis infected and not infected with *S. pseudintermedius*. Thirteen unique proteins after *in-gel* digestion of the areas of interest were identified. However, only four seem promising candidate therapeutic targets (Table 19): the selection of these proteins was made on the basis of the predicted surface localization and/or crucial function in bacterial pathogenesis or survival (e.g. adhesion) (Delany, Rappuoli & De Gregorio, 2014).

Table 19. Characteristics of the 4 immunogenic antigens from *S. pseudintermedius* FMV5819/10 strain.

Protein (abbreviations)	Subcellular localization	Functional category
Fibronectin-binding protein SpsD	Cell wall	Pathogenesis associated proteins
Lipoprotein, putative	Unknown	Unknown
ATP synthase subunit alpha AtpA	Membrane	Energy metabolism
Enoyl-[acyl-carrier-protein] reductase [NADPH] FabI	Membrane	Fatty acid and phospholipid metabolism

One of these highly immunogenic proteins identified was SpsD, which has already been described by another study as a candidate therapeutic target (Bannoehr et al., 2011). The gene encoding this protein was also highly transcribed by the MRSP strain as detected by RNA-seq. This could mean SpsD is essential for the pathogenesis of MRSP infections. The predicted lipoprotein had an unknown location however it has a predicted cleavage site, meaning that it may be secreted via the Sec pathway. Furthermore, lipoproteins are a major class of cell surface-exposed proteins in many bacterial pathogens and play critical roles in nutrient uptake, antimicrobial resistance, adhesion, protein secretion, and other functions (Sutcliffe & Russell, 1995). These molecules have been the targets of vaccine research for many pathogenic bacteria, including *S. aureus* (Vytvytska et al., 2002). The other two proteins, AtpA and FabI, were associated with metabolism and seem to have crucial roles in bacterial growth (Balemans, Vranckx, Lounis, Pop, Guillemont, Vergauwen, Mol, Gilissen, Motte, Lançois, Bolle, Bonroy, Lill, Andries, Bald & Koul, 2012; Schiebel, Chang, Lu, Baxter, Tonge & Kisker, 2012), which makes them approachable target candidates.



5 Conclusions

The main findings of this thesis were:

a) Horses and calves can carry MRSA in their nostrils, acting as reservoirs of these bacteria, which can potentially be transmitted to humans and companion animals.

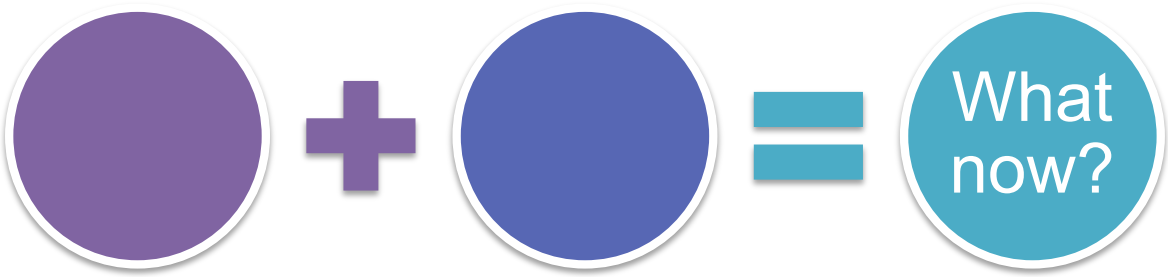
b) There was a clear dissemination of specific MRSA clones (CC5, C22 and CC398) between animals, the environment and humans in close contact. Furthermore, isolates of the clonal lineages associated with pets (CC5 and CC22) harboured specific sets of virulence genes and a lower number of resistance genes than isolates of the clonal lineage associated with livestock-animals (CC398). Active surveillance programs, not only detecting MRSA in livestock-animals, but also in companion animals, are urgently needed.

c) Biocides appear to be a reliable antiseptic option against MRS from horses and MRSP from small animals. Even in the presence of bacterial efflux determinants, like *qac* genes, the in-use recommended concentrations of the biocides were much higher than the *in vitro* MBC.

d) The increase over time of *mecA*-positive and multidrug-resistant staphylococcal strains is worrying. Several MRS clonal lineages circulating in human hospitals and in the community were found in this study, suggesting that companion animals can become accidently infected with highly successful human MRS clones or that these clones are not host specific. Thus, companion animals can act as reservoirs of important bacterial clones and genes of human origin, perpetuating the transmission cycle of MRS.

e) An MRSP isolate highly expressed a number of virulence and regulatory genes when compared to MSSP that could explain its high and fast adaptation. Biofilm formation appears to have an important role in *S. pseudintermedius* pathogenesis, although there were differences in the mechanisms triggering biofilm production in MSSP and MRSP.

f) Thirteen unique proteins were identified after *in-gel* digestion of highly IgG immunogenic areas, with four antigenic proteins showing promising features for vaccine development against *S. pseudintermedius* pyoderma.



6 Future work

1. Establish national surveillance programs, not only detecting MRSA in livestock-animals, but also in companion animals.

The trends of antimicrobial resistance in animals is worrying, especially in small animals. Active surveillance programs, not only detecting MRSA in livestock-animals, but also in companion animals, are urgently needed.

2. Study the dynamics of methicillin-resistant coagulase-negative staphylococci in the human, animal and environmental communities as reservoirs of virulence and antimicrobial resistance mechanisms.

Other staphylococci, especially MRCoNS are neglected as important pathogens and reservoirs of antimicrobial resistance genes in human and veterinary medicine. Studies on MRCoNS in people, animals and environment will give an oversight on the prevalence of these bacteria. Molecular epidemiology will provide insights into the dynamics of MRCoNS in the human, animal and environmental communities and will also give information on phenotypic characteristics, such as virulence and antimicrobial resistance mechanisms. Comparison of SCC*mec* elements of MRCoNS and MRSA could give comprehension on how often does SCC*mec* transfer occurs. These studies could help understand the epidemiology of MRCoNS and therefore lead to the implementation of control strategies to prevent the dissemination of these bacteria in the animal and human population.

3. Assess virulence determinants as prognostic factors of *S. pseudintermedius* infection in the dog.

Despite the multitude of studies on virulence factors, there are only a few studies correlating these with clinical characteristics *in vivo*. In human infections caused by *S. aureus* there are studies conducted that identified certain virulence factors as more often associated with certain clinical characteristics such as persistence or morbidity (Jarraud et al., 2002). In animals, and in particular in *S. pseudintermedius* in dogs, this has not been studied thoroughly. Further studies using the novel “omic” approaches could help identify these crucial virulence factors for a better treatment regimen against *S. pseudintermedius* infections as well as for better prognosis.

4. Determine the efficacy of the *S. pseudintermedius* tetravalent vaccine in experimental and natural infection in the dog.

With the four proteins that were identified we could include them in a tetravalent vaccine to be subsequently tested for its ability to induce protection in a canine skin model of infection.

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